

Science policy by conjuror's hat

The British government's penchant for sudden reorganization is plainly matched by its capacity instantly to formulate goals for research and to choose snappy titles for them, but when will it learn to think what it is doing?

WHOEVER said that the British government's decision to move the still-new Office of Science and Technology from the Cabinet Office to the Department of Trade and Industry (DTI) would be bad for British science (see *Nature* 376, 103; 1995)? The opposite, of course, is the truth. Only last week, Mr Ian Taylor, a minister at DTI, was able decisively to put critics of the new arrangements firmly in their place: British science can now rejoice in the new slogan "EQuAL" (where the cases of the letters are apparently deliberate) and there are to be six prizes, worth £150,000 in total, for university departments and academic research institutes able to demonstrate successful collaboration with industry. DTI says the lucky winners of the competition can use the funds (£35,000 for a first prize, £15,000 for a second, with separate competitions in 'science' and 'engineering') "to improve the technology transfer services they offer". What carping critic can ask for more?

EQuAL is something else again, a demonstration that the research enterprise cannot fail to benefit from the marketing skills of Britain's all-purpose sponsor of British industry. As an acronym, EQuAL breaks new ground. While most examples of this genre stand for noun phrases (NATO and CERN, for example), EQuAL stands for a whole sentence: "Extend Quality Life". (Quibbling grammarians may complain that it should be written "EQuAL!"; it is to be hoped that it is not too late for DTI to consider that amendment.) But, of course, EQuAL is more than a mere acronym. According to DTI, it is a "new blue print (*sic*) for Science Engineering and Technology policy in Britain".

Policy

What, the world will be asking, is that new policy? Critics of successive governments' neglect of science are an impatient crew and even now will be harrying DTI for guidance on this important but (to DTI) peripheral matter. Do they not have the courtesy to let ministers decide in their own good time, with deliberation? Surely those same critics are precisely those demanding more deliberation in the handling of science policy? Have they no consideration for the ministers pitchforked into EQuAL by the instant reorganization of OST two weeks ago? And what, except triteness, is wrong with the declaration of principles attributed to Taylor last week by DTI? "Both quality of life and wealth creation are important, and they are not mutually exclusive. Improved health gives better quality of life and

improved quality of life can bring economic benefits." That may not say much about British policy on CERN, or the lack of industrial investment in innovation, but who can quarrel with it?

Scope

Sadly, the misanthropic critics are unlikely to be satisfied with this unexceptional remark or with the other declarations that follow it. Taylor's argument is that "strategic aims help us to ensure that we have core strengths", that "our aim is to help people remain active for as long as possible" in which cause "we must all work together". He foresees a partnership between "fundamental genetics, physics and chemistry" with "long distance diagnosis using leading-edge technology and new patterns of work and leisure". There is nothing wrong with this blueprint of a dynamic gerontocracy except that, like the reorganization two weeks ago, it has been pulled like a rabbit from a conjuror's hat. There is nothing to suggest that this, rather than other goals, will generate the "core strengths" needed to revitalize the British economy. When another conjuror comes along, there will doubtless be another rabbit, perhaps called "TYPES!" (for "Teach Young People Extra Skills!"). Nobody would be able to quarrel with that, either. There would even be scope for "long distance teaching using leading-edge technology and new patterns of play and learning".

Vacuity on this scale is one of the critics' long-standing fears of what would happen to British science if it fell into the hands of civil servants who did not know enough to care and under the eye of ministers persuaded that "leadership" is their role, whatever its direction. (Taylor's yearning for dynamic maturity for the British population is comparable with Mme Edith Cresson's love-affair with the electric car in her new role as the commissioner for research at Brussels: a whim turned into policy by fiat.) It is all the more ironical that this should be happening in the wake of the great foresight exercise in which all sectors of the British economy were asked to specify their expectations of science in the decades ahead, but whose value is not tested yet.

EQuAL and the accompanying rhetoric will offend most working scientists for several reasons. To the extent that the slogan is a gimmick, it conflicts with the seriousness of their purpose, which is to learn more about the working of the world we live in. Even those searching for



new Alzheimer's genes or designing new prostheses will not have been dignified by last week's announcements. But the prospect that the pattern of British research will increasingly become a patchwork of goal-directed programmes requiring that people's imagination should be servant to other people's curiosity is the obvious danger.

There is nothing wrong with that if the feasibility of the goals has been agreed in advance with those who will do the work. During its three brief years of independence, OST had begun to win a reputation for being prepared at least to listen. Indeed, the new establishment at OST had begun to behave as if it meant what it was saying about the need for a constructive partnership with the research community. That was one of the objectives of the Foresight exercise, after all: the particular objectives defined by all those Delphi groups might mean very little, but the method by which they had been achieved would create solid relationships between researchers and their beneficiaries. The research community would have had its say, usually influential. Without agreement, the terms on which most researchers are employed in Britain will implicitly have been changed. They will work less effectively, and with less enthusiasm.

Pity, in particular, Professor Robert May, who was appointed Chief Scientific Adviser to the British government and the effective head of OST only a few days before the displacement of his office to DTI. May is one of Britain's most energetic and ingenious researchers; he has held a Royal Society research professorship at the University of Oxford for the past several years. A robust Australian, he is not the kind who would have agreed to move to OST if he believed he would be unable to make a decisive mark. (Nor is he the kind who would relish pity.) But on the face of things, he has been cheated. Whatever DTI may say, OST is no longer influentially at the heart of government. May's chance of coordinating the whole of the government's policy on science from a base at DTI is negligible. In the circumstances, Taylor should not be too surprised if May fails to turn up for work on 1 September, the agreed starting date, but chooses to sue the government for constructive dismissal instead. As things have turned out, that may be the best that May can do for British science. □

Clinton neutron promise

The future for large physics facilities in the United States looks bleak.

WHEN the Clinton administration finally put the Advanced Neutron Source (ANS) out of its misery in February (see *Nature* **373**, 460; 1995) it made a promise to US neutron users in general and to the Oak Ridge National Laboratory in Tennessee, which was to have hosted the ANS, in particular. The idea of a new neutron source "is not dropped at this point", Jack Gibbons, Clinton's science adviser and a former Oak Ridge physicist, said at the time. "We have

shifted the focus to accelerator-based technology" from the reactor-based ANS, explained Gibbons. And the source would still be built at Oak Ridge, the president's budget proposal said.

This turnaround placed Oak Ridge in an odd position, since no plan, however tentative, existed to build an accelerator-based neutron source there. The laboratory is only now ready to start work on one, and will receive \$16 million over the next two years to complete a conceptual design.

The requirements to be met by the Oak Ridge designers will be set by the Department of Energy subject to the advice of its Basic Energy Sciences Advisory Committee (BESAC). The United States would like to match the proposed 5-MW European Spallation Source, but the panel has been told that a maximum of \$1 billion will be available — enough for a 1-MW source, experts say. BESAC may fit the quart into the pint pot by proposing a smaller source, that could be upgraded at some later date.

In contrast, the Argonne National Laboratory in Illinois already has a comprehensive design for an accelerator-based source in hand: newly printed, it runs to 800 pages. But it was conceived as an upgrade to existing Argonne facilities. The Illinois laboratory is just completing construction of the \$800-million Advanced Photon Source (APS), and convention says it cannot have both.

Los Alamos, meanwhile, with a pragmatic eye to the Department of Energy's dismal budget prospects, is offering to upgrade its neutron scattering centre for less than \$100 million.

The Department of Energy's brief to BESAC is disappointingly sparse. It asks the panel to investigate updates to existing reactor-based sources, but implies that this might be done "within existing budgets". And it tells the panel to stick to the Oak Ridge site, come what may. In doing so, it comes dangerously close to wasting the time of BESAC and Oak Ridge by asking them to pursue preparations for a \$1-billion machine that neither this administration nor Congress is prepared to build.

Under a Department of Energy plan drawn up in 1987 — a golden age, in retrospect — Argonne got the APS, Lawrence Berkeley Laboratory in California got the Advanced Light Source, the Brookhaven National Laboratory on Long Island, New York, got the Relativistic Heavy Ion Collider — and Oak Ridge was to get the neutron source. This 'Buggins' turn' system of allocation has a quite straightforward logic: the even distribution of Energy Department largesse assures a wide distribution of political support for the department and its science programmes.

The practice — common to most branches of the US government — has always been easy to mock, and is now under sustained attack in Washington from those who brand it "politics as usual". But it has served science and the United States extremely well. If the new majority in Congress is really determined to bring it to an end, it is hard to see how any large physics facility will be built. in the United States in future. □

Dispute over 'threshold' explosions could disrupt test ban negotiations

Paris. International efforts to negotiate a Comprehensive Test-Ban Treaty (CTBT) are in the balance because of proposals by nuclear weapons states to exempt low-yield nuclear explosions. Non-nuclear weapons nations seem prepared to abandon efforts to agree on a treaty altogether rather than to accept the proposals in their current form.

A comprehensive test-ban is widely considered to be the price that nuclear weapons states promised to pay earlier this year for agreement among non-nuclear states on an indefinite extension of the Nuclear Non-Proliferation Treaty (NPT).

But whereas the non-nuclear states have until recently seemed ready to agree to a sufficiently comprehensive ban to encourage disarmament as well as non-proliferation, they fear that the new proposals would drop disarmament from the CTBT's goals.

The controversy over the CTBT — now being negotiated at the Conference on Disarmament at Geneva — centres on closed talks among the permanent five nuclear weapons state members of the United Nations Security Council, known as the P5. These talks are intended to fix the threshold below which explosions would be exempt from the ban, the main issue within the so-called "activities not prohibited" section of the treaty.

The official position of the United States is that only hydronuclear experiments (HNEs) with yields below 1.8 kg equivalent of TNT should be exempt from the ban. Such small HNEs are not very useful for designing weapons, but they are widely accepted as useful for testing the safety of stored weapons. What distinguishes HNEs from full-blown nuclear tests is that the chain reaction is stopped before a full-yield explosion occurs.

Last month, however, William Perry, the US defence secretary, announced that the United States had reopened discussions on the level of this threshold. The Pentagon has since argued that tests with yields as high as 500 tonnes should be exempt from the ban, although the White House has, for the present, rejected this proposition.

The US announcement followed a demand by France that the threshold be fixed at 200–300 tonnes. The United Kingdom, whose official position calls for a threshold of 45 kg, is also said in some circles to be keen on raising this to 500 tonnes. "I think the hawks in the nuclear weapons states are working together on this to undo the treaty", says Christopher Paine from the Natural Resources Defense Council (NRDC), based in Washington, DC.

The proposals have been greeted with

Japanese demonstrators: should concerns focus on maximum levels for tests rather than France's plans to resume testing?

dismay by non-nuclear states, which see them as evidence that the P5 states are not genuinely committed to a comprehensive test-ban. India, the *de facto* leader of the non-aligned states, has retaliated by proposing that the treaty should ban all tests involving fissile material, which would even outlaw HNEs with yields of only a few kilograms.

India's proposal represents a hardening of the position of the non-nuclear states. Until recently, these seemed ready to accept not only 1.8-kg-yield HNEs, but also a request by Russia that HNEs of up to 10 tonnes should be allowed. Russia has difficulty controlling HNEs, and its case for what is often referred to as a 'whoops factor' has been widely accepted as justified.

According to Rebecca Johnston, the Geneva representative of the London-based Verification Technology Information Centre (VERTIC), India is sending a "strong signal to the nuclear weapons states that if they do not agree to a comprehensive test ban, there will be no test ban at all".

Johnston claims that non-nuclear states have hardened their positions because they feel the P5 have failed to respect the "gentlemen's agreements" given to win their consent to the NPT. The ink on the NPT had barely dried, she points out, before China exploded a bomb, France announced its decision to resume testing, and the United States reopened the debate over thresholds.

Speaking at Geneva earlier this month, Boutros Boutros Ghali, the secretary general of the United Nations, warned that failure to reach agreement quickly on nuclear ►

France keeps the experts guessing

Paris. France's proposal to exempt low-yield explosions from the Comprehensive Test-Ban Treaty (CTBT) (see above), combined with its recent decision to resume nuclear testing, has provoked a vigorous debate about the goals of its nuclear weapons programme.

Jacques Chirac, the French president, has stated that one or two of the eight proposed tests are needed to qualify the TN75, a miniaturized, 100-kilotonne warhead for the M5 intercontinental ballistic missile (ICBM) designed for the next generation *Triomphant*-class submarines. Another two tests are needed to maintain the existing French stockpile, he says, and a further four for calibrating simulation techniques.

French officials also argue that they need a new generation of weapons that would be less "sensitive" to ageing and could be remanufactured reliably. They point out that France gets less information from its tests than the United States because of technical problems, a claim supported by reports

from the US Central Intelligence Agency.

Christopher Paine, a senior research associate at the Natural Resources Defense Council (NRDC) in Washington, DC, says that France "seems to have a reasonable case" for testing the TN75 warhead. But he contests claims by France that further tests are needed to ensure the reliability of the stockpile — although admitting that outside access to data on the state of French weapons makes it difficult precisely to evaluate French arguments in favour of testing.

But many suspect that France is developing tactical warheads. Indeed, although the government says that its policy of nuclear deterrence remains unchanged, some parts of the French military argue that variable-yield designs are needed, including weapons with lower yields. But one senior military official, who supports this position, argues that the aim is not to develop battlefield weapons, but to provide a deterrent more suited to the post-Cold-War geopolitical situation. **D. B.**

►testing would “undermine” efforts by the international community to achieve disarmament. “The question of the threshold is a much bigger issue than the French decision to carry out eight more tests”, says Patricia Lewis, head of VERTIC.

Conventional nuclear weapons could not be tested fully within the proposed threshold of 500 tonnes. But such tests would provide nuclear weapons states with data that would give them added confidence in a new design, says Lewis.

She adds that the thresholds being proposed are in the range that is “crucial for weapons design work”. In a recent report, the NRDC also argued that such thresholds would allow study of thermonuclear fusion, and the yield of new “boosted” fission designs.

In a curious twist in the dispute over the scope of the CTBT, a senior military official said last week that if France is allowed to carry out its planned tests in the South Pacific, it would back down on its demand that low-yield tests be exempt from the CTBT.

“If France goes ahead with the tests it will not demand a threshold in the CTBT,” he says. “We are willing to drop the threshold as a gesture of self-restraint aimed at helping international cooperation.”

His remarks are consistent with comments attributed to Jacques Bouchard, the head of the military applications division of the French Atomic Energy Commission (CEA), in a report by the NRDC and the Federation of American Scientists (FAS), a Washington-based lobby group. Bouchard was quoted as having said last year that “the alternative to France conducting a series of nuclear tests would be to insist on a CTBT that would allow tests of at least 100 tons”.

Declan Butler

Physicists reveal glimpses of Japan's atomic bomb effort

Tokyo. As the fiftieth anniversary of the end of the war in the Pacific approaches, details are beginning to emerge of the little-known efforts by Japanese scientists to develop an atomic bomb during the Second World War.

Tatsusaburo Suzuki, an 83-year-old former researcher who was sent by a military institute in 1944 to work on the project at the Institute of Physical and Chemical Research (RIKEN), told a press conference in Tokyo last week that Japan had the necessary expertise to build the bomb but lacked sufficient resources.

“Towards the end of the war, some experts thought it would take us about 100 years to build the bomb,” he is reported to have told the Tokyo meeting. “I was of the opinion that if we spent 100 times more in research efforts, we could have developed the bomb in one year.”

According to a leading Japanese physicist who was a student at Osaka University during the war, there were three groups in Japan working on what was called the ‘uranium bomb’. One group at RIKEN was headed by the institute director, Yoshio Nishina, an eminent nuclear physicist who had studied under Niels Bohr in Denmark. A second group at Osaka University was led by Seishi Kikuchi, another eminent physicist. A third group was at Kyoto University.

Two approaches were pursued, the electromagnetic separation of uranium 235 and separation by a thermal diffusion process. For the second of these, Kikuchi’s group at

Osaka built a tall double pipe system extending through several floors of the building. But it was not very successful, and according to one member of the group, it switched to research on a high-powered magnetron before the end of the war.

Very little is known about the research carried out at RIKEN. All records were destroyed after the war, and a spokesman for the institute says “we have no materials”. But it is known that the institute was the target of US bombing raids because of its work on nuclear research, and that it suffered severe damage during a raid in April 1945.

But most Western experts conclude that Japan was far behind the United States, Great Britain and Germany in attempting to develop a bomb.

Nevertheless, even after the war RIKEN suffered badly for its war-time efforts. In November 1945, engineers from the US occupation forces dismantled two cyclotrons at the research institute and dumped them in Tokyo Bay. Cyclotrons at Kyoto and Osaka universities suffered similar fates. This was a major setback for Japan’s high-energy physics research from which it took decades to recover.

In theory, the cyclotrons could have been used to separate uranium isotopes. But in practice they would have been unable to produce sufficient quantities for a bomb, and the cyclotron destruction was widely condemned by the US high-energy physics community at the time. **David Swinbanks**

Congress saves Cassini, but targets infrared astronomy mission

Washington. Only eight days after its threatened demise, the international Cassini mission to Saturn was rescued last week in the US Congress, as the House of Representatives Appropriations committee restored the project’s full funding request of \$249 million for 1996.

The committee also reversed a subcommittee’s decision to close three field centres belonging to the National Aeronautics and Space Administration (NASA) (see *Nature* 376, 203; 1995), and called for an agency restructuring study instead. But the Mission to Planet Earth and the proposed Space Infrared Telescope Facility (SIRTF) both suffered budget cuts as NASA rides a roller-coaster through the congressional appropriations process.

In restoring the Cassini money deleted by a subcommittee, the full committee acknowledged the project’s importance and virtually ensured that it will remain on track for a 1997 launch. But the money had to come from somewhere, and the

committee therefore took \$339 million out of a \$1.34-billion request for Mission to Planet Earth, which includes the Earth Observing System (EOS).

It also reduced the budget for the airborne SOFIA infrared astronomy programme by nearly half, to \$28.7 million. While the Gravity Probe-B relativity experiment received full funding at \$51.5 million, SIRTF was given no money for next year. NASA had asked for \$15 million to continue studying the project in 1996, with spacecraft development to begin in 1998 and launch planned for 2002.

The House Science committee, chaired by Robert Walker (Republican, Pennsylvania), which produced a NASA authorization bill last week, also recommended scaling back EOS and deleting SIRTF funds in 1996. According to staff members, the committee’s intention is not to kill SIRTF but merely to delay it until funds become available as other expensive missions such as Cassini

get closer to their launch dates.

The project is seen as the infrared entry in NASA’s suite of ‘great observatories’. These include the Hubble Space Telescope, the Compton Gamma Ray Observatory and the Advanced X-Ray Astrophysics Facility (AXAF). Advisory committees from the National Academy of Sciences have consistently given SIRTF high priority among proposed astronomy projects. But its high cost (\$560 million), its lack of international participation and the fact that it is not yet under way has made it a tempting target for budget-cutters.

According to NASA managers, without the 1996 money the launch would slip by a year, and some of SIRTF’s scientific return would be compromised, as it would not be able to conduct as many coordinated observations with other space observatories. A launch in 2003 would miss most of the opportunity to overlap with the Hubble Space Telescope, as well as any overlap with AXAF. **Tony Reichhardt**

Neutron source plans face uncertain future

Washington. The US Department of Energy (DOE) has asked a top-level advisory committee to reassess the future needs of US researchers for access to neutron sources, following the cancellation earlier this year of the proposed \$3-billion Advanced Neutron Source (ANS).

But a letter sent last month by Martha Krebs, director of the Office of Energy Research, requesting the advice of the DOE's Basic Energy Sciences Advisory Committee (BESAC), sets no deadline for a response.

Furthermore, say officials at DOE laboratories, the cash-strapped department is in no rush to fill the gap left by the cancellation of the ANS. They add that, as a result, neutron users may have to wait ten years or more before a new neutron source comes on line.

Neutrons are increasingly in demand from condensed-matter physicists, materials scientists, structural biologists and others studying the structure of molecules. Scientists in all these disciplines are able to use both the steady stream of neutrons generated by reactors and the short bursts created by proton accelerators through a process known as spallation — generation of neutrons by the acceleration of protons into a heavy metal target — for carrying out different types of experiment.

Furthermore, neutron science is one of the few capital-intensive research fields in which the United States seriously lags behind Europe. According to a BESAC report published in 1993, Europe has three times as many neutron scientists as the United States — and, since 1970, has spent five times as much money on neutron facilities.

The 1993 report was prepared by a sub-panel of the advisory committee chaired by Walter Kohn of the University of California at Santa Barbara. It concluded that the energy department should give priority to the construction of the ANS reactor, and then back a new 1-MW spallation source.

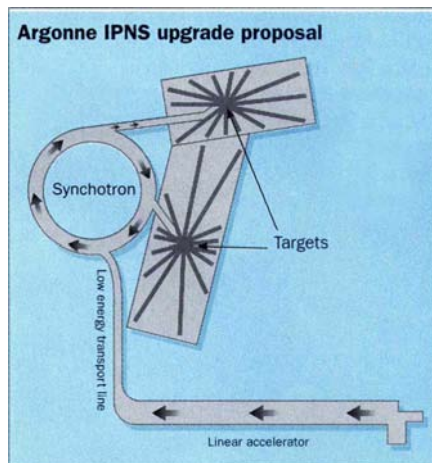
But BESAC now faces the task of sorting the debris left by the cancellation of the ANS into a coherent neutron programme for the department.

In her letter, Krebs asks BESAC chairman Carl Lineberger, professor of chemistry at the University of Colorado at Boulder, to evaluate possible upgrades of existing reactor-based neutron sources at the Oak Ridge National Laboratory (ORNL) in Tennessee, and the Brookhaven National Laboratory on Long Island, New York. She also asks the panel for its advice on the type of accelerator-based neutron source that could be built at ORNL for "about one-third" of the estimated \$3-billion cost of the ANS.

Oak Ridge, which was to have hosted the ANS, has already been identified by the DOE as its preferred site for a new spallation source — even though it has no design

in hand. The laboratory is due to receive \$8 million in the next financial year, which starts in October, to draw one up. It also has an ageing reactor source, the High-Flux Isotope Reactor (HFIR), which will require major refurbishment to extend its useful life.

By contrast, the Argonne National Laboratory in Illinois has a well-advanced design



for a 1-MW spallation source, which it would like to build as an upgrade to its existing Intense Pulsed Neutron Source (IPNS). This would cost around \$750 million — \$175 million less than elsewhere because it would be able to make use of existing buildings, according to Bruce Brown, director of IPNS.

Brookhaven also has a design for a spallation source. But its top priority is a long-overdue, \$30-million instrumentation upgrade for its 30-year-old High Flux Beam Reactor. Like HFIR, this reactor's pressure vessel will need replacing in the near future.

And the Los Alamos National Laboratory in New Mexico is proposing a new target for its existing neutron scattering centre, arguing that this could provide the United States with an internationally competitive neutron spallation source three years from now for less than \$100 million.

Given these different demands, some difficult choices will have to be made. "I don't think there is \$1 billion out there for a community of 1,500 people," says Roger Pynn of the Los Alamos Neutron Scattering Experiment. "If BESAC has any sense of realism, they are going to recommend doing something with existing facilities."

But Krebs's request to BESAC — as well as the department's immediate budget plans — implies that a new spallation source will eventually be built at Oak Ridge. Except that the embattled department has no money, and not much enthusiasm to proceed. If everything goes to plan, says Iran Thomas, acting director of the DOE's Office of Basic Energy Research, the source could be producing neutrons by 2002. But, he says, that completion date is "wildly optimistic".

Colin Macilwain

HIV scandal hits Bombay blood centre

New Delhi. A blood bank in Bombay operated by the India Red Cross Society (IRCS) has been closed down following a government decision to conduct an inquiry into charges that the centre had supplied blood infected with HIV to hospitals in the city between 1992 and 1994.

The medical officer in charge of the blood bank has already been removed from his post, and more officials are expected to lose their jobs. The affair has caused concern throughout India, as the IRCS is intended to set national standards for blood safety.

The Bombay blood bank, which supplies 15,000 units of blood a year — about one-third of the city's requirements — is one of the 16 blood-testing centres making up the National AIDS Control Organization (NACO), set up in the region with an US\$87-million grant from the World Bank.

Eight out of 30 children suffering from thalassaemia who are registered with the blood bank have become HIV-positive, and the Maharashtra state government has told its health officials to track down all known recipients of the tainted blood.

The affair came to light when J. N. Banarjee, chairman of the IRCS subcom-

mittee on blood transfusion services, examined the records of the blood bank. These showed that HIV-positive blood had been supplied to at least 10 city hospitals, including one that specializes in transfusion services.

In addition, there have been suspicions that employees of the blood bank, rather than discarding infected blood, have been selling it on the black market created by an overall shortage of blood supplies.

India has more than a thousand blood banks, half of them run privately. But these generate only 1.95 million units a year — less than half the country's need. An expert committee has estimated that India needs a further investment of about US\$90 million to upgrade its blood banks to acceptable levels of hygiene.

According to NACO, the control of AIDS in India will be difficult to achieve if blood supplies are not made safe. Out of 1,108 cases of AIDS reported up to 1 June, it estimates that 11.1 per cent had contracted the disease from infected blood.

NACO also claims that 30 per cent of blood is collected from professional donors, many of whom are HIV-positive.

K. S. Jayaraman

France seeks revised role in space station

Munich. Prompted by France, the council of the European Space Agency (ESA) agreed last week to consider a new proposal for Europe's contribution to the international space station. Rather than the planned space laboratory, which would be built by Germany and Italy, it is proposing a crew transfer and re-entry vehicle (CTRV), which would probably be built in France.

This new proposal was first announced by François Fillon, French minister for information technology and telecommunications, at the launch of the military satellite Helios in French Guyana earlier this month. But it could delay a final decision on Europe's exact role in the space station.

Until the beginning of this year, ESA's proposed contributions to the space station had three elements: a crew rescue vehicle (CRV), an Automated Transfer Vehicle (ATV) to transfer cargo to the space station, and the European laboratory module, known as the Columbus orbital facility (COF).

But after Germany and France, the two major participants in the programme, demanded a halving of the planned ECU3.5-billion (US\$4.62-billion) costs between 1996 and 2000, it became impossible to maintain all three elements. In March, ESA therefore put forward a revised proposal from which the CRV had been removed.

In response, France said that it was no longer prepared to meet its previous commitment to the programme, despite being allocated prime responsibility for the low-cost ATV. In contrast, Germany, which would lead the COF project, was happy with the new proposal.

But ESA has still not been able to raise the full costs of this revised proposal (see *Nature* 374, 586; 1995). Only ECU1.4 billion of the ECU1.8 billion required between the years 1996 and 2000 has been definitely committed — and this assumes that Italy will be able to pay its promised ECU300 million that it would like to contribute. This failure has given France the opportunity to request ESA to study a proposal that it finds more acceptable.

ESA has now developed a plan to reduce costs further, to ECU1.5 billion, by extending the construction timetable of the two remaining elements. But ESA's council has also accepted a French request to cost a second proposal, based on combination of the CRV and the ATV.

The two options reflect a fundamental difference in philosophy over the space station between Germany and France — both driven by their own industrial interests.

IMAGE
UNAVAILABLE
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REASONS

All aboard: the Crew Rescue Vehicle (CRV) would form the basis of a new space station component.

France sees the CTRV as an opportunity to develop the techniques required to establish a lead in re-entry technology.

It hopes that the vehicle could be a forerunner of the space plane Hermes which it had planned to build before financial difficulties hit ESA. France also claims that independence in re-entry technology should be an important political aim for Europe.

Germany, in contrast, supports the COF as a way of participating fully in the use of the space station for scientific experiments. It also argues that the construction of the laboratory represents a considerable technological challenge for European industry, primarily in the development of data management and life support systems.

If the COF is built, as ESA is currently proposing, Europe would be allocated 7.8 per cent of the whole station's experimental use, and this would increase to 9 per cent if ESA wins responsibility for providing a regular boost for the station back into its

correct orbit. If the COF were to be replaced by the CTRV, it would be entitled to only about 3 per cent of use, according to calculations made by the German space agency, DARA.

France appears unconcerned at this prospect. "The French scientific community is not that interested in using the station; it is more interested in astronomy and planetology, which exploit satellite data, than microgravity," says André Lebeau, president of CNES, the French space agency. An additional advantage would be that ESA's contributions to the common operational costs of the space station would also fall if its utilization share was lower.

Germany does not have unqualified support from its own scientific community, which in the past has questioned the cost-effectiveness of the experimental opportunities offered by the station, given that its own strengths lie in astrophysics. "We have a small scientific population with interest in microgravity," concedes Jan-Baldem Menicken. But, accepting that money spent on the station would never in any case be redirected to them, scientists are no longer arguing the case.

Germany's position is now firmly behind the COF-ATV option: "it fulfils all the agreed goals of utilization and cargo transportation," says a DARA spokesman. Moreover, it has the support of the United States and has already been integrated into the intergovernmental draft agreement on space station participation (between Japan, Canada, United States, Russia, Europe). "The United States has told us that the laboratory is our entry ticket to the international space station", he says.

The CTRV option must be developed very quickly if it is to have a serious chance of being considered. While the COF-ATV option has financially binding agreements from industry for all of its parts, equivalent costings for the CTRV have not been carried out. Yet it has a very strict deadline, as it must be available in 2002 when the ageing Soyuz rescue vehicle is phased out. (The United States and Russia are negotiating its replacement).

ESA will now cost out France's proposal during August, and member states will spend September deciding which of the two competing options should be brought to October's ESA ministerial meeting, when a final decision on European participation in the space station must be decided.

Publicly, France and Germany are stressing heavily their interest in reaching agreement as soon as possible. But given their different viewpoints, ESA could be forced to ask the ministers to make the final choice — which could itself result in a deadlock that could postpone any decision.

Allison Abbott

Technology office: now it's up, now it's down

Washington. The Office of Technology Assessment (OTA), which provides the US Congress with analysis of science and technology issues, has returned to the critical list after the Senate rejected by 54 votes to 45 an amendment to keep the threatened office alive.

Last month, the House of Representatives unexpectedly voted by 220 to 204 to retain the OTA's functions within the Library of Congress (see *Nature* 375, 711;

1995). Senators who voted to close the 170-strong office last week included friends of science such as Carol Moseley-Braun (Democrat, Illinois) and Mark Hatfield (Republican, Oregon).

The Senate vote could result in the death of the OTA when the budget bills of the two houses are combined in September, as virtually all of the senators and congressmen taking part in the conciliation conference are pledged to close it. C. M.

Defence physicist takes on top post at Australia's CSIRO

Sydney & London. Malcolm McIntosh, chief of defence procurement in the UK Ministry of Defence, is to return home to Australia to head the troubled government science agency, the Commonwealth Scientific and Industrial Research Organisation (CSIRO).

But as McIntosh cannot take up the \$A200,000-a-year (US\$145,000) appointment until the beginning of next year — and to allow efforts to continue the search for solutions to the CSIRO's recent well-publicized internal problems — Peter Cook, the Minister for Industry, Science and Technology, has taken the unusual step of appointing the organization's acting chief executive, Roy Green, as chief executive officer.

McIntosh, who is 49, holds a PhD in physics from the Australian National University, and started his career as a research scientist in the Australian Weapons Research Establishment. He has held his current post in the United Kingdom for five years, dealing with a number of politically charged decisions that have included negotiations over the purchase of Chinook helicopters and, most recently, restructuring the Eurofighter programme.

Before taking up that post, he was the most senior civil servant in Australia's Department of Industry, Trade and Commerce. He was chosen from a shortlist of 14 candidates, and says of the CSIRO that "clearly the institution is all about excellence in science, and I would want to see it compete with the best in the world".

But McIntosh remains discreet about his views on current problems within the organization — in particular those arising from communication difficulties between management and staff (see *Nature* 372, 585; 1995) — saying "I do not at present know enough to be able to comment on how to solve them".

Green has been the CSIRO's acting chief executive since the departure of the former chief executive, John Stocker, in March. As such, he has had to deal with revelations about the poor state of the organization, as well as discontent among its scientists.

Green had previously said that any major changes to the organization — such as the recommended removal of a layer of management — would have to await the arrival of its new chief executive. He had been due to retire in November, but will now remain until McIntosh arrives.

CSIRO's problems were recently highlighted by an incident in which five senior executives were virtually forced to read out a letter of apology over the wording of a submission made by their staff to the board.

Mark Lawson & David Dickson

Breeder plans are dented as thermal reactor support falls

Tokyo. Japan's electric power companies have decided to drop their support for construction of an advanced thermal reactor (ATR), a move which may deal a fatal blow to plans to build commercial fast breeder reactors (FBRs) in the early decades of the next century.

On 11 July, the nine members of the Federation of Electric Power Companies asked the government to scrap a long-standing plan to build a demonstration ATR, intended to pave the way for commercial FBRs. The reactor was to have been built in Ohma, in Aomori Prefecture, at the northern tip of Japan's main island of Honshu.

According to the federation, soaring costs for the proposed 606-MW ATR have made the project commercially unviable. The original cost estimate was ¥396 billion (US\$4.56 billion) in 1984, compared to the latest government estimate of ¥580 billion.

Instead of an ATR, the members of the federation now propose building a much

commission nearly thirty years ago in 1966 as part of an overall strategy leading to the development of FBRs. The reactor, which uses heavy water as a moderator and a mixed uranium-plutonium fuel, was intended to consume part of Japan's growing stockpile of plutonium before the introduction of commercial fast breeders, scheduled to take place around 2030. Construction of the ATR was due to begin in 1998, and it was to have come into operation in 2004.

Nearly ¥300 billion has already been invested by the government and industry in developing the ATR over the past three decades. This figure includes ¥68 billion spent on constructing a prototype, named Fugen, that has been in commercial operation since 1979 (see picture). In the current fiscal year alone, nearly ¥5 billion has been set aside for the ATR project by the STA.

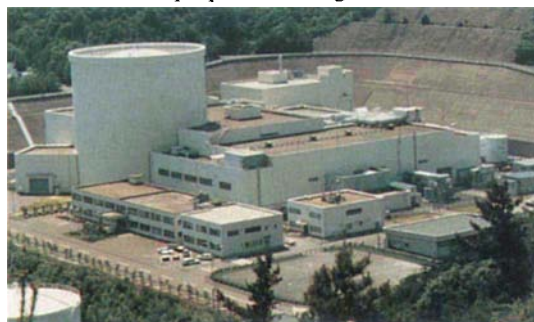
But the Federation of Electric Power Companies estimates that electricity from the Ohma demonstration reactor would cost

¥38 a kilowatt, three times as much as that produced by a conventional light-water reactor. With the government planning to deregulate the electric power industry from next year, the industry felt it could not continue to support the project. Furthermore, new technology means that light-water reactors can now burn mixed plutonium-uranium fuel, and the ATR has therefore lost its main purpose.

STA officials claim that abandoning the ATR would not directly affect the development of FBRs.

Indeed, FBRs are not dependent on ATRs in a technical sense. But industry observers say the federation's decision suggests that the electric power industry is unlikely to support the development of commercial FBRs, which are more expensive to build and operate than ATRs.

David Swinbanks



Fugen reactor: this prototype now seems unlikely to be followed by full-scale advanced thermal nuclear station.

cheaper conventional light-water reactor at Ohma. This would have more than twice the power of the proposed ATR, and would fulfil the same role of burning up some of Japan's growing stockpile of plutonium derived from reprocessed fuel.

Officials of the Ministry of International Trade and Industry (MITI) and the Science and Technology Agency (STA) have been trying to play down the significance of the federation's announcement. They say that the fate of the ATR will be decided by the Atomic Energy Commission, not by the electric power industry.

But last week, Makiko Tanaka, the outspoken female politician who heads the STA and chairs the commission, effectively killed the ATR by announcing at a press conference it is "only natural" that industry should pursue profits. "Even if the Atomic Energy Commission and the STA discuss the matter, I don't expect the federation to change its mind," Tanaka said. Without industry's support the project cannot proceed.

The ATR project was launched by the

Biotech tax set-back

London. Britain's Secretary of State for Health has refused to support pleas from the biotechnology industry for special tax status. In an interview with the *Financial Times*, Stephen Dorrell said he supported investment in these fields. But he was "not in favour of tax shelters".

The remarks have caused consternation among Britain's biotechnology industry. Paul Haycock of the Biotechnology Industry Association, described Dorrell's remarks as "very discouraging". Others said he was speaking out of turn, as the taxation of research is the responsibility of the British Treasury. □

Embryo research faces a renewed ban in US

Washington. Following intense pressure from anti-abortion lobby groups, a committee of the US House of Representatives last week voted for a complete ban on research using human embryos at the National Institutes of Health (NIH).

The move has raised concern that the NIH, worried about the political fate of its overall budget request, may not risk either encouraging or carrying out such research during the term of the current Congress.

NIH neither conducts human embryo research in its own laboratories nor funds researchers elsewhere to do so. But it has for several months been on the brink of releasing ethical guidelines for such research,

based on the recommendations of an expert advisory panel (see *Nature* 371, 370; 1994).

The guidelines would provide guidance to both clinicians and laboratory scientists, and would open the door for NIH to begin its own research under certain restrictions. In particular, the panel recommended that research on embryos should be limited to the first 14 days after fertilization, a point at which cells are undifferentiated and the primitive streak has not yet appeared. It also agreed that, in special circumstances, oocytes could be fertilized for the purpose of research.

The advisory panel's recommendations were passed to Harold Varmus, the director

of NIH, last September. Many believe that his reason for not releasing them is the fear that the guidelines could provoke a backlash from conservative legislators in a Congress keen to make deep cuts in the biomedical research agency's budget.

Similar concern appears to have made the research community — unlike either clinicians or patients' groups — reluctant to try to protect human embryo research from the proposed ban, approved as an amendment to the \$61-billion appropriations bill for the Departments of Labor, of Health and Human Services and of Education.

Under the bill as approved by the House Appropriations committee, the NIH would be spared the deep cuts proposed on education, job-training and health programmes. Instead, it would receive a 5.7 per cent increase in 1996.

Research using live human embryos has some vocal opponents in Congress. Jay Dickey, (Republican, Arkansas), for example, who drafted the amendment along with Roger Wicker (Republican, Mississippi), described such research as "orwellian".

Dickey's proposal was among several added to the bill with the intention of curbing funds for abortion or family planning. He claims a further effect is likely to be felt in moves to reinstate a ban on federally funded fetal tissue research imposed by the previous Republican administration but lifted by President Bill Clinton in 1993. "We want to reclaim that ground," said Dickey.

The Dickey amendment banning embryo research was nearly undermined by an alternative proposal drafted by John Edward Porter (Republican, Illinois), which reflected an executive order issued in December last year by President Clinton (see *Nature* 372, 490; 1994).

In particular, Porter's amendment would have permitted federal funding for human embryo research, provided the embryos used had not been created specifically for research purposes. But a tied vote of 26:26 meant that the proposal was not adopted.

The decision has been criticized by at least one member of the advisory panel. "The irony is that the goal [of research using human embryos] is to help people have babies and avoid abortions," said Mark Hughes, chief of reproductive and prenatal genetics at Georgetown University.

The lack of both ethical guidelines and funding from NIH for such research means that it will continue to be funded privately. The research is conducted mostly by infertility clinics, and focuses primarily on ways to increase the success rates of implantation; it will continue to be driven largely by profit, and will continue to lack peer review. Hughes says that he had hoped that NIH's guidance "would spill over into the private sector".

Adrienne Appel

California drops affirmative actions

San Francisco. Faculty members at the University of California (UC) were last week assessing the likely impact of a decision by the university's governing 'Board of Regents' to discard the use of 'affirmative action' in hiring and admissions.

Those who had worked for years to help develop opportunities for women and minorities in science formed part of the protest at the symbolic message of the new policy — even though they differed over whether it would mean fewer women and minorities in science-related departments.

"The harm is social harm," says Francisco Ayala, chairman of the American Association for the Advancement of Science, and a professor of evolutionary genetics at UC, Irvine.

The regents voted 14:10 against continuing affirmative action policies in admissions, hiring and contracts. University officials said the change would be felt most deeply at the undergraduate level, as the hiring of faculty members and post-graduates is left to individual departments.

Furthermore, the resolution approved by the regents excludes any changes that would lead to the loss of federal or state funds to the schools. All federal contractors are required to set goals for recruiting women and minorities.

As a result, Ayala says the student population is likely to remain largely unchanged, while at the faculty level affirmative action policies have taught recruiters to look harder in non-traditional places.

"We have come so far that I don't think [recruiting] will be affected," he says. But he adds that, while women have made progress in science and other fields, there are still few minority faculty members.

Nevertheless, Ayala accepts that the regents' decision is likely to have a significant symbolic impact, complaining that the regents did not seem to feel that the make-up of the student population should

reflect that of the state of California. "If we reduce diversity, it will be very unfortunate, for all sorts of reasons," says Ayala.

Sherrie Wilkins, past president of the Palo Alto chapter of the Association for Women in Science, felt the decision would inevitably harm the ability of women and minorities to succeed in science, even though the university is still bound to affirmative action in hiring.

"It won't have an impact today or tomorrow, but it will have a long-term impact," says Wilkins, who serves as a hiring consultant for bioscience companies. She adds that women and minorities remain underrepresented in science, and will have to continue to struggle for equal representation for a long time.

She claims that, while making hiring and admissions decisions based solely on merit makes sense on the surface, many studies have shown that 'merit' lacks objectivity. "There are not enough people who feel that the genders are the same in terms of intellect, capability and logic," says Wilkins.

A university report presented to the regents two months ago estimated enrolment among ethnic groups would change dramatically without affirmative action. The report found that African-American enrolment could drop by 40 to 50 per cent to just 2.5 per cent of the total student population. African-Americans make up 7 per cent of the state's population.

Latino enrolment was predicted to fall by 10 per cent. As a result, said the report, Latinos will make up just 12 per cent of the University of California's population, even though they constitute 30 per cent of the population of the state.

In contrast, Asian-American enrolment was predicted to climb by 15 per cent to 25 per cent. As a result, Asian-Americans would constitute 30 per cent of UC students, even though they make up only 10 per cent of the population.

Sally Lehrman

Prizes fail to quell disquiet over UK policy

London. Britain's Department of Trade and Industry (DTI) announced last week that it is to offer six annual prizes, with a total value of £150,000, to reward projects aimed at encouraging technology transfer and linking universities to industry.

It also, somewhat more obscurely, announced what it described as "a new blueprint for science engineering and technology policy in Britain" under the title *Extend Quality Life* — or *EQaL* (*sic*) — which it defined as a way of integrating "the entire spectrum of scientific, technological economic and social activities".

Both initiatives, which took many UK research administrators by surprise, were launched at a press conference organized in response to widespread concern about the government's controversial decision to transfer the Office of Science and Technology from the Cabinet Office to the DTI (see *Nature* 376, 103 and 206; 1995).

Government officials continue to defend the move. At the press conference, Ian Taylor, the new junior minister for science and technology, justified it on the grounds that while Britain remained strong in curiosity-driven research, "the transfer of ideas to industry is very patchy". Moving the OST to the DTI — and thus closer to industry — was therefore a "logical step" that puts the

infrastructural groupings in the world".

But, even if the initial outcry has died down, many scientists remain convinced that pressures will increase on the science base to meet the short-term needs of industry, and that politically, science has been demoted. "The move seems to show that the government feels scientists should be on tap, and not on top," said one observer.

As further evidence of this, in a reshuffle of cabinet committees, the science subcommittee was last week disbanded, and its functions merged into a new ministerial committee on economic and domestic policy and competitiveness. The committee will be chaired by Michael Heseltine, former head of the DTI and a strong believer in harnessing science firmly to the needs of British industry, who is now deputy prime minister.

Cabinet responsibility for science now rests with Ian Lang, the new president of the Board of Trade. According to Taylor, Lang will "make sure that the science voice will be heard at the government level". But there are reports — which DTI officials deny — that Lang was a less than fully active participant in a meeting last week of the government's Council for Science and Technology, which he now chairs. He was also reported, in an interview with the *Daily Telegraph*, to have told Taylor to take over as minister for science and technology "to keep the scientific community happy".

Whether the reports are true or not, last Friday's press conference seemed to be more of a damage limitation exercise than an attempt to set out any carefully crafted new policy initiatives. Its defensive tone was evident in a statement issued by Taylor, arguing that the government "has in no way downgraded science and engineering".

The statement also emphasized that "quality of life [is] not a poor relation" — an apparent reference to the concern generat-

ed by the initial announcement of the shift of the OST to DTI which described the mission of the science base merely in terms of its potential contribution to national "wealth creation".

EQaL, said Taylor, is a reflection of this new commitment. He said that it required the integration of activities ranging from "fundamental molecular science (genetics, physics, chemistry) to long-range diagnostics and new patterns of work and leisure". And Taylor added that some of the funds being awarded to support the pursuit of strategic goals outlined in the recent Technology Foresight exercise would be directed towards EQaL's components.

But lacking any clear mandate, specified goals or organizational details on how EQaL will be administered — or even any commitments to new funding — many were left wondering whether the "new initiative", which appears to have had little detailed discussion in science advisory circles, will prove much more than an attempt to paper over the cracks in the government's reputation.

Certainly the Royal Society remains lukewarm. While refraining from overt criticism of the government's moves, the society's council issued a statement last week saying that it was keen to hear further details of the thinking behind the restructuring.

There is a growing feeling that some of this nervousness will disappear if the government is able to secure the continued existence of the House of Commons Select Committee on Science and Technology, now threatened with being subsumed into the committee that covers the whole of trade and industry. The Royal Society is one among many groups that have expressed a desire to see the select committee's survival. A decision is expected in October when parliament meets for the beginning of its new session.

David Dickson



activities of the research councils for whom OST is responsible "on a bigger platform".

Taylor also defended the fact that, as a result of the move, the chief scientific adviser (CSA) to the government, a post to be taken up at the beginning of September by Robert May of the University of Oxford, will now operate from the DTI. "To detach the CSA from the rest of OST would be great mistake," he said.

There was further support from Sir William Stewart, the government's chief scientific adviser from 1990 until last month. In a newspaper article, Stewart wrote that the shift to the DTI was a "shrewd move" that could lead to "one of the strongest

Equality in sight for contract research staff

London. Contract research staff at British universities will be employed on terms equivalent to their full-time peers, according to a draft 'concordat' released last week by the Committee of Vice Chancellors and Principals (CVCP).

The document proposes equal pay, pensions and leave for fixed-term contract researchers, whose numbers have risen fourfold to 18,000 over the past 12 years. It also agrees to recognize previous experience when arranging salaries and career development.

"It is essential that universities can recruit from the best researchers," says Kenneth Edwards, chairman of the CVCP. But the concordat advises contract staff not to view a fixed-term

research post as the first step to full-time academic employment. "This is realistic only for a minority," it says.

The Association of University Teachers (AUT) welcomed the concordat as a belated step in the right direction. But the AUT's national president, Peter Breeze, pointed out that it failed to recommend an end to waiver clauses on unfair dismissal and redundancy.

The consultative document, which stems from the 1993 science White Paper, was drafted by a group led by the Office of Science and Technology, with input from the research councils, the Royal Society and the CVCP. A final version is expected to be approved by the end of the year.

Ehsan Masood

Corruption and appointment

SIR — Angel Pestaña's letter (*Nature* 375, 626; 1995) outlines the difficulties in procuring stable positions for those engaged in scientific research in Spain. Pestaña uses the Spanish Scientific Research Council (CSIC) as a model to explain the situation, but the CSIC can probably be considered as the élite of Spanish research and the real situation is therefore much worse. An additional problem that he mentions only briefly is "secular endogamy in the universities". The 'Ley de Reforma Universitaria' (LRU), approved by parliament in the 1980s, was supposed to end such endogamy, while decentralizing the Spanish universities, creating new stable positions and facilitating the movement of personnel within the different universities. The theory was excellent, but in practice the LRU has failed to reform the corrupt university establishment.

Appointment committees for a permanent university lectureship consist of two members of the university and three outsiders from different universities. Almost invariably there is an 'official' candidate for the position, who has normally been in the job on a contract basis for a number of years; the 'official' candidate is almost inevitably the one appointed, sometimes with complete disregard for the greater suitability and higher standards of other applicants. Furthermore, due to the bureaucratic nature of the process, applying and preparing for a single lectureship costs an enormous amount of money and time (nine months is not unusual), with almost no guarantee of a fair examination.

Human capital investment should certainly be a priority in science policy in Spain. But a serious modernization of the universities, with a new, less bureaucratic, more pragmatic, flexible and fair system for university and other scientific appointments is much needed.

Vicente Rodilla

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Climate treaty and equity

SIR — Your leading article "Conflicts of interest on the greenhouse" (*Nature* 374, 483; 1995) raises some critical questions on climate change, several of which, as you point out, were not asked at the recent Berlin conference. But it also contains a few inaccuracies and points in need of clarification; these, in part, explain why such questions remain unaddressed.

Your statement that "in due course, equity requires that the same rules should apply to all emitting countries" seems to conflict with the principle of "common but differentiated responsibilities" embodied in the climate treaty. It would in fact be inequitable to impose the same rules on all countries, given the developed world's historical role in greenhouse gas emission — although the developing world, with a larger prospective role, will eventually require specific obligations.

Second, you say that China and India are classified in Annex II of the treaty: Annex II includes developed and not developing countries. But for the latter to take on further commitments will require more financial and technical assistance from the former than we have seen so far.

Finally, your well-taken point that issues of "entitlement" and per capita emissions are on the horizon reminds us that the climate treaty must ultimately be placed within the context of "sustainable development". As a prime example, rapidly developing China may become the world's leading emitter by 2020; yet its per capita emissions today are one-ninth those of the United States. Herein lies the unavoidable challenge of equitably including now-poor countries in a global warming regime.

Nevertheless, your hope that another conference will tackle these issues may be fulfilled. The Berlin mandate requires that negotiations for a protocol be conducted "with urgency", and the climate treaty itself allows for additional meetings under "extraordinary circumstances". Coming events may very well provide such a context.

Seth Dunn

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Job for the boy?

SIR — Grant applications, seminars, congress proceedings and reports of any kind: scientists are flooded with hard-to-refuse requests from colleagues and peers for ready-by-tomorrow papers that would take months to be properly digested and delivered. It might be useful to know, therefore, what to do in such circumstances. So we would like to propose St Expeditus as the patron of anyone facing deadlines. Although *Nature's* readers certainly include people of every race, sex, age and creed, we hope that, in the name of the independence and universality of science, we will be forgiven if this saint belongs to the Christian faith.

Although nothing is known about his life and the circumstances of his martyrdom, St Expeditus is honoured on 19 April together with St Donatus Hermogenes and 22 fellow Christians who were killed in

Melitenes (Cappadocia, Turkey)¹. He is usually represented as a standing Roman soldier who crushes under his left foot a raven croaking the Latin word *cras* (tomorrow): this bird, indeed, was regarded by the ancients as the emblem of endless procrastination². St Expeditus holds the crown of martyrdom in one hand and indicates a clock with the other; in later images the clock is replaced by a cross bearing the Latin word *hodie* (today), meaning that one should never put off until tomorrow what can and must be done today.

Veneration for St Expeditus dates back at least to 1554, as demonstrated by a manuscript listing a church dedicated to him in the bishopric of Périgueux (Périgord, France)³. In southern Germany the saint is still honoured and is represented in a wealth of engravings and paintings, the oldest of which, dated 1759, bears the caption *Patron deren so ihre geschäft glücklich vollenden wolen desen Fest so den 19 April* (Patron of those who wish to bring their business to a happy end; his day is 19 April), while another reads *Heute, noch, nicht morgen erst, Sei gethan, was Gott begehrt* (What the Lord commands has to be done today, right now, not tomorrow)⁴. Since the mid-eighteenth century, the cult of St Expeditus has also been recorded in Sicily, especially in the maritime cities on the eastern coast of the island, such as Messina and Acireale, where the saint was proclaimed secondary patron and invoked by merchants and seamen for quick and safe clearing of their business (*negotiorum et expeditionum patronus*). Under a different, but corresponding name (St Minas, derived from the roots of *expedite* [speedy] and *arakahas* [fast-arriving]), this saint may also be identified in the Armenian Martyrology⁵.

Although such a veneration smells of superstition and originates probably from an easy pun on the saint's name, in popular belief St Expeditus has become the enemy of any postponement, and the person to whom to pray to obtain an immediate grace. The saint seems to have enjoyed a successful career, and can be rightly elected as patron of all desperate scientists asking for out-of-this-world help in meeting deadlines.

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1. Sauget, J.-M. in *Bibliotheca Sanctorum* 5, col. 95 (Istituto Giovanni XXIII della Pontificia Università Lateranense, Città Nuova Editrice, Roma, 1964).

2. Cahier, P. C. *Caractéristiques des Saints dans l'Art populaire* 1, 256 (Poussielgue, Paris, 1867).

3. Lavielle, J. *Semaine religieuse*, Périgueux, 16 décembre 1905.

4. Kaul, L. *La Civiltà Cattolica* 56, 718–727 (1905).

5. Aucher, G. *La Civiltà Cattolica* 57, 448–460 (1906).

More muddle over the Hubble constant

A new calculation makes it likely that Type 1a supernovae will be more useful standard candles for estimating the distance scale of the Universe, but discordance is unlikely to be banished soon.

CONFLICT and confusion about the value of the Hubble constant continue. For many years, it has been a source of acute embarrassment to cosmologists that there appear to be two distinct and discordant ranges in which observational estimates lie. On the one hand, there are values lying in the region of $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$, on the other, there is a cluster of estimates almost twice as large, in the region of $80 \text{ km s}^{-1} \text{ Mpc}^{-1}$. (In these expressions, 'Mpc' stands for the non-SI unit megaparsec, which is a million parsecs or nearly 3 million light-years.)

The discordance is important because the Hubble constant, which is literally a measure of the rate at which the expansion velocity increases with increasing separation between our Galaxy and another, also sets the physical scale for the Universe and therefore, by inference, its age. The smaller values of the Hubble constant suggest a greater age, certainly more than 15 billion years. The larger values suggest a smaller age, perhaps 10 billion years or less.

Notoriously, the smaller ages for the Universe raise problems, at least for those who believe that the modelling of stellar structure has become a precise art-form. For it is well known that some globular clusters in our Galaxy contain stars whose age is estimated to exceed 12 billion years, and which may be as great as 16 billion years. That belief is plainly at variance with the larger estimates of the Hubble constant.

That conflict was sharpened just under a year ago, with the publication of two estimates of the Hubble constant based on observations of the variable stars called Cepheid variables in galaxies of the Virgo cluster (M. J. Pierce *et al.* *Nature* **371**, 385; 1994 and W. L. Freedman *et al.* *Nature* **371**, 757; 1994). Both measurements, based on stars in different members of the Virgo cluster, gave values for the Hubble constant in the upper range. On that basis, there seemed little doubt about the supposed conflict between the age of the Universe and that of the oldest stars in our Galaxy.

But the argument is far from being settled. For one thing, last year's results depend on arguments for telling the placing of the galaxies in which Cepheid variables were observed in relation to the cluster as a whole. That is one important source of uncertainty. Another is the difficulty of interpreting the variations of the observed brightness of the distant galaxies from which periods of oscillation must

be derived.

Luckily, the second set of observations last year derived from the Hubble Telescope. The hope must be that there are more and even better observations to come. In short, the wait for a definitive answer to the question whether there is a conflict between the age of the Universe and that of the oldest known stars may not be long.

Yet the research network keeps generating numbers at the other end of the bimodal spectrum of the Hubble constant. The latest is due to a group of physicists at the University of Oklahoma and is interesting for its own sake as well as for the support it offers those who would like the talk of conflict to go away (P. Nugent *et al.* *Phys. Rev. Lett.* **75**, 394–397; 1995). What Nugent and his colleagues have done is to take the handful of high-quality observations of Type 1a supernova explosions and to calculate their absolute luminosity.

If the description is in any way relevant to the explosion of a star, a Type 1a supernova explosion is an almost well-ordered happening. The total energy output of the event reaches a maximum in two to three weeks, after which it decays with half-lives corresponding to the radioactive decay times of ^{56}Ni and ^{56}Co of 8.8 days and 111 days respectively.

While there is still apparently room for argument about the kinds of stars that blow up in this way, one favoured explanation is that such a supernova is formed from a white dwarf which is part of a binary system, and which accretes enough material from its companion to spring into life again. Because at least the core of a white dwarf is degenerate in the sense of quantum mechanics, the gravitational forces at the surface will be considerable, the accreted matter will be greatly compressed and thermonuclear reactions may be ignited in shells of material near or even at the surface, involving elements even heavier than helium, carbon for example. The supernova explosion takes the form of the ejection of a shell of matter. Whether or not these explosions are capable of generating elements heavier than iron is an open question, but there is no doubt that iron is one of the end products.

The essence of what Nugent and his colleagues attempt is to calculate the link between the maximum luminosity of a supernova and the time between the initial explosion and maximum brightness in two different ways. First, they tackle the trans-

port of radiation and matter in a stellar atmosphere thrown off by the initial explosion (which is not child's play, but a relativistic problem). On that view, the length of time to maximum brightness is a rapidly increasing function of the amount of energy released in the explosion, which is what would be expected; modest explosions would quickly fizzle out.

Then they work through the consequences of supposing that the expansion of the atmosphere is driven by the successive radioactive decay of ^{56}Ni and ^{56}Co into ^{56}Fe . These processes are supposed to deposit their energy in the exploded atmosphere of the star directly. At 1.1 MeV for the decay of a nickel nucleus, simple arithmetic shows that there is enough energy to keep even a supernova glowing provided that the amount of nickel is a substantial fraction of the mass of the Sun.

The outcome is of general interest. If radioactive elements play the part foreseen for them, the time to maximum brightness for all Type 1a supernovae must lie between 15 and 20 days. By the authors' account, the agreement between these predictions and the behaviour of the known and well-observed supernovae of this type is as good as can be expected. The crucial point, for cosmologists, is that the same methods make it possible to calculate the absolute luminosity of a particular supernova at its peak, so providing the 'standard candle' required for fixing distances in the Universe.

The practical difficulties are nevertheless serious. Supernovae of this type do not occur every day, and then only in distant galaxies. Telling how long it takes for an explosion to reach its peak is usually a matter of searching through other people's chance observations to find when the explosion happened. Even so, there seems little doubt that the new argument will help to make a tricky class of observations more tractable.

But, of necessity, there is little chance that the muddle about the value of the Hubble constant will melt away as a result. And, sadly, the prospect that there will quickly be a dramatic increase of the distance over which people can observe these and other standard candles is not bright. A stroke of luck might put things right, but that cannot be prearranged. The pity is that while the discordance persists, cosmologists will not know which way to turn on questions like the reality of the Big Bang.

John Maddox

Smart transcription factors

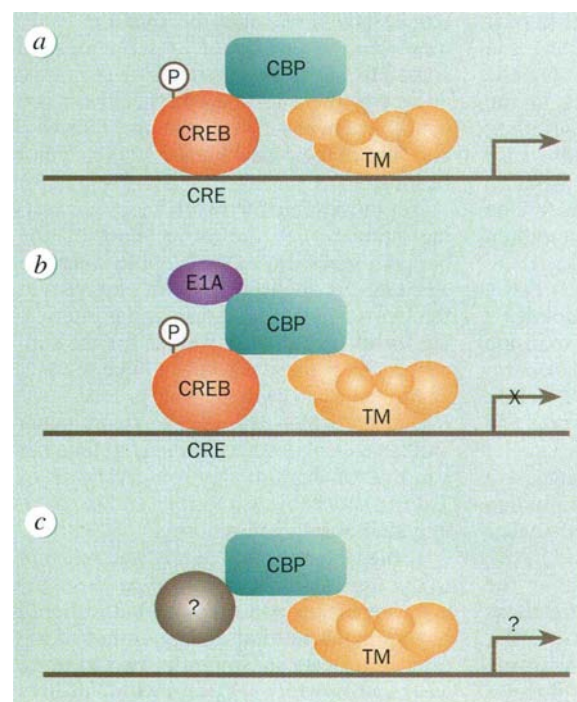
Gabriella D'Arcangelo and Tom Curran

On page 348 of this issue¹, Petrij *et al.* establish a link between cognitive function and the regulation of gene expression by showing that Rubinstein-Taybi syndrome (RTS) is associated with mutations in the transcription co-activator known as CREB-binding protein (CBP). RTS is a human genetic disease characterized by mental retardation and physical abnormalities that include broad thumbs and broad big toes. It is estimated to account for as many as 1 in 300 cases of institutionalized mentally retarded subjects. In addition to the principal clinical features, a wide variety of other developmental defects, most notably craniofacial malformations and neoplasms, are often associated with RTS². The disease is transmitted in an autosomal dominant manner and it has been mapped to chromosome 16p13.3, the location of the gene encoding CBP.

CBP was first described as a protein that binds to the phosphorylated form of the CREB transcription factor and increases the expression of genes containing cyclic AMP-responsive elements (CRE) (see figure)³. An increase in the level of intracellular cAMP activates protein kinase A, which in turn phosphorylates CREB on serine 133, thus promoting association with CBP and stimulating CRE-dependent gene expression. In addition to this cAMP signal-transduction pathway, CBP may also mediate transcriptional activation in response to growth factors. It is closely related to the adenovirus E1A-associated protein p300, and *in vitro* studies indicate that both CBP and p300 can mediate E1A-induced gene repression and cell immortalization, as well as CREB-dependent gene activation⁴. CBP has also been shown to bind to TFIIB, a component of the basic transcription machinery, suggesting that it provides a physical and functional link between activated signal transduction pathways and the transcription machinery⁵.

The majority of biochemical studies have so far implicated CBP in the transmission of cAMP-dependent signals as a result of interaction with CREB. In several animals, *Aplysia*, *Drosophila* and mice, the cAMP pathway has been shown to be important for at least one aspect of brain function, the establishment of long-term memory⁶. This function has now been extended to CREB itself using transgene and gene-knockout approaches^{7,8}. Indeed, overexpression of CREB in *Drosophila* significantly enhances the formation of long-term memory⁹. However, memory abnormalities have not been reported in RTS patients who suffer from mental retardation. Furthermore, neither severe developmental anomalies nor the

neoplasms associated with RTS are seen in CREB-deficient mice. Thus, the results of Petrij *et al.*¹ point to unexpected links between CBP (and presumably the cAMP signalling pathway), intellectual ability, tissue development and cell growth. The divergence of phenotypes resulting from



Regulation of gene expression by CREB-binding protein (CBP). *a*, CREB can bind to DNA in a non-phosphorylated state; phosphorylation on serine 133 recruits CBP, providing a link to the transcription machinery (TM). CRE, cyclic AMP-response element. *b*, Adenovirus E1A binds to CBP and represses gene expression. *c*, CBP may regulate transcription through interactions with as-yet unidentified proteins.

defective CREB and CBP also indicates that these two proteins may be more functionally independent than *in vitro* studies had suggested.

How did the CBP-RTS connection come about? Petrij *et al.* were tracking down the gene responsible for RTS by positional cloning, and narrowed the critical region down to a 150-kilobase stretch of DNA in the 16p13.3 chromosomal region by mapping a series of deletions and translocation breakpoints. Using this stretch of genomic DNA as a probe, they cloned coding sequences highly related to mouse CBP. In one patient, CBP messenger RNA appeared to be smaller than it should be, and in two others point mutations gave rise to truncated CBP proteins. Petrij and colleagues argue that the reduced amount of normal CBP protein in the hemizygous condition is responsible for RTS. However, a few caveats need to be considered.

First, deletion of the CBP gene has not yet been clearly demonstrated in genomic DNA from RTS patients and it is possible that other genes may be present in the RTS critical region, particularly considering the map of that region is incomplete. Readers may recall that *Cell* recently published a pair of papers that implicated two different genes in the genetic disease spinal muscular atrophy¹⁰, but it turns out that only one of these genes is likely to be responsible for the disease. This case reminds us that genetic correlation, however compelling, does not constitute proof of causation.

Second, the decrease in the level of normal CBP protein predicted for hemizygous patients has yet to be demonstrated. This piece of data is critical to the authors' claim that decreased CBP gene dosage is responsible for the disease. In our view, although CBP is a most promising candidate gene for RTS, its identity still needs to be confirmed.

The idea that RTS is a consequence of reduced CBP gene dosage (haploinsufficiency) is based on RTS cases resulting from hemizygous deletions of a chromosomal region that does not appear to be subject to imprinting¹. This type of transmission, although rare, has been described for mutations involving metabolic enzymes, structural proteins and transcription factors¹¹. Examples of transcription factor haploinsufficiencies include aniridia (of the gene *PAX6*)¹² and Greg syndrome (of *Gli3*, a member of the *GLI-Kruppel* gene family)¹³. Changes in transcription factor levels will alter their activity or specificity by affecting the composition of complexes involving these proteins. For example, Krüppel mediates transcription activation at low concentrations and transcription repression at high concentrations, when the propensity for

- Petrij, F. *et al.* *Nature* **376**, 348–351 (1995).
- Hennekam, R. C. M. *et al.* *Am. J. med. Genet. suppl.* **6**, 17–19 (1990).
- Chrivia, J. C. *et al.* *Nature* **365**, 855–859 (1993).
- Lundblad, J. R. *et al.* *Nature* **374**, 85–88 (1995).
- Kwok, R. P. S. *et al.* *Nature* **370**, 223–226 (1994).
- Frank, D. A. & Greenberg, M. E. *Cell* **79**, 5–8 (1994).
- Bourtchuladze, R. *et al.* *Cell* **79**, 59–68 (1994).
- Yin, J. C. P. *et al.* *Cell* **79**, 49–58 (1994).
- Yin, J. C. P. *et al.* *Cell* **81**, 107–115 (1995).
- Lewin, B. *Cell* **80**, 1–5 (1995).
- Fisher, E. & Scambler, P. *Nature Genet.* **7**, 5–7 (1994).
- Ton, C. C. T. *et al.* *Cell* **67**, 1059–1074 (1991).
- Vortkamp, A., Gessler, M. & Grzeschik, K.-H. *Nature* **352**, 539–540 (1991).

homodimer formation is increased. Thus, a modest quantitative change in a transcription factor can result in a qualitative alteration in target-gene expression.

Several important questions concerning both RTS and CBP still remain, in view of the poorly understood nature of the anatomical and physiological defects in RTS patients. It will be interesting to see whether a mouse hemizygous for the CBP gene recapitulates the developmental and learning dysfunctions associated with RTS. The relationship between CBP and

p300 is also intriguing: as noted by Petrij *et al.*, p300 cannot substitute for a reduced CBP-gene dosage in RTS patients, so it is important to determine whether this is due to different temporal or spatial patterns of expression, or to functional differences between the two proteins.

Although the defects in RTS are likely to be developmental in origin, the association with CBP offers a novel therapeutic target. In many cases patients can be identified at birth by the morphological characteristics of RTS. Although gene

therapy is impractical at present, an alternative strategy could be to identify agents that increase CBP activity, thereby alleviating some of the disease symptoms. Drugs that act on the cAMP signalling pathway might also prove to be beneficial to RTS patients, perhaps even enhancing memory and cognitive function. □

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SUPRAMOLECULAR CHEMISTRY

New ribbons and threads

Leonard F. Lindoy

THERE has been a growing trend among chemists to construct larger molecular entities by using smaller components as 'building blocks'. Now, a group headed by Fritz Vögtle in Bonn has used this strategy to prepare novel molecular 'ribbons' composed of up to seven repeating units¹, as well as new examples of 'threaded' molecules (rotaxanes)².

It is now around 200 years since the foundations of modern chemistry were laid down by the likes of Antoine Lavoisier and John Dalton. Their ideas about atoms and the way they combine set in train developments which ultimately led to procedures for obtaining a myriad of new molecules. Increasingly, as with the work of Vögtle's group, much of this synthetic activity falls into the realm of supramolecular chemistry — broadly the chemistry of large multicomponent molecular assemblies in which the component structural units are held together either by covalent linkages or by one or more weaker (non-covalent) interactions. The latter commonly include hydrogen bonds, dipole-dipole attractions and/or stacking of π -electron clouds.

In earlier work, the Bonn group was successful in producing short ribbons such as molecule **1** (a in the figure; see ref. 3). However, all attempts to extend the series to include molecules containing more than four benzene rings were unsuccessful, even though longer linked-ring systems have been reported previously⁴; the latter are of a different type made up entirely of fused six-membered rings.

In the work just published¹, Vögtle and co-workers describe a new iterative strategy (a in the figure) that has allowed the preparation of the desired extended ribbon molecules, in this case containing 5–7 benzene rings. These ribbons may be considered to be composed of repeating [3,3]metacyclophane units, with the 'outside' appended tosyl groups contributing to their excellent solubility — an important consideration if these extended

molecules are to be incorporated into even larger supramolecular systems in the future.

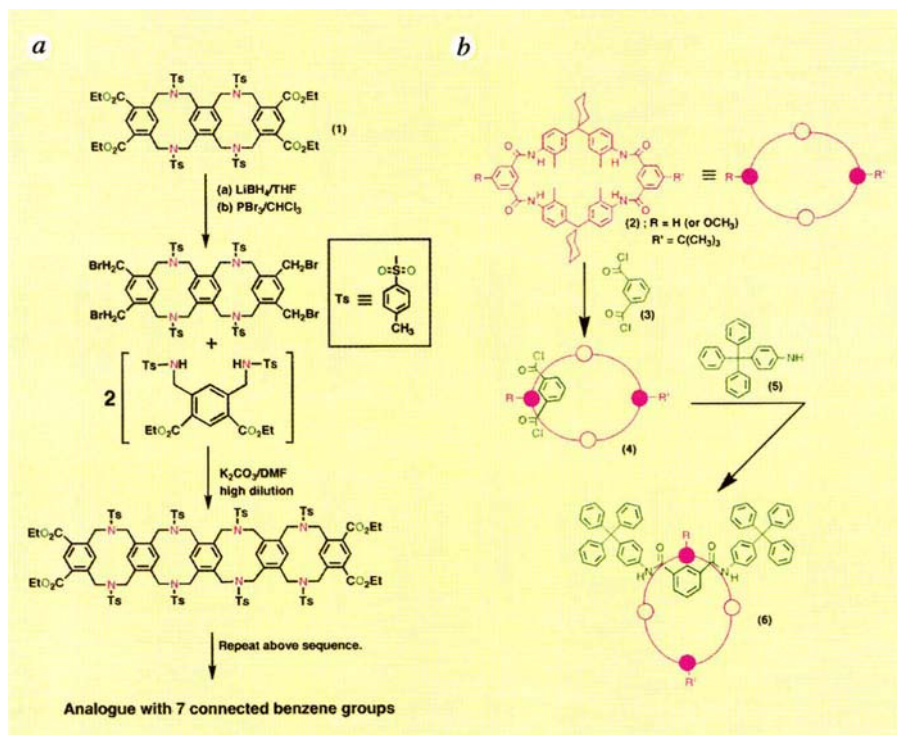
The seven-ring system represents the longest sequence yet reported of benzene rings connected in this manner. Moreover, using the new procedure, the synthesis of even longer strings now seems feasible.

Single-crystal X-ray analysis of the five and six benzene ring systems indicates that *syn* (folded back) arrangements of the repeating units occur in each case such that the benzene rings align themselves on top of, and roughly parallel to, each other. Evidence indicates that the remaining seven-ring system adopts a similar 'fluted' arrangement. The present structures re-

semble those adopted by an otherwise linear molecule that has been shown to rearrange spontaneously to a 'pleated' architecture in water⁵ — reflecting enhanced interaction in this solvent between alternately positioned electron-rich and electron-deficient units along the chain.

Following from these studies, Vögtle has anticipated the future production of new families of nanoscale ribbons, belts and tubes, the latter two by linking the ends of suitably modified ribbons to yield structures that resemble in general form those reported previously by Stoddart and co-workers⁴. The cyclic products will probably act as selective receptors for trapping small molecules of suitable dimensions in their central cavities.

The Bonn group has also used a supramolecular template effect to produce a new type of rotaxane² — a category of supramolecular compounds in which a cyclic molecular 'wheel' is threaded on a separate 'axle' — with the axle then being



a, Representative iterative procedure for preparing extended ribbons, as in the synthesis developed by Vögtle and colleagues¹. b, The self-assembly of a new rotaxane.

capped at both ends with bulky groups to lock the wheel in place.

The procedure used starts with the amide-containing tetralactam macrocycle (molecule **2**, *b* in the figure) which was prepared by a conventional ring-closing condensation reaction^{2,6}. The addition of the isophthaloylic derivative (**3**) to a solution of (**2**) results in a host-guest interaction between these species to yield the corresponding intermediate (**4**). Molecular models indicate that there is a snug fit between host and guest in **4**, with the guest being orientated at right angles to the host. The host and guest appear to be held together by a combination of weak π - π interactions and $\text{NH}\cdots\text{O}=\text{C}$ hydrogen bonds. The final step involves the condensation of two bulky amine groups (**5**) to cap both ends of the 'axle' giving (**6**), the first amide-based rotaxane. Clearly, the synthesis proceeds by means of a combination of molecular template and self-assembly processes, as simultaneous reaction of **2**, **3** and **5** in the ratio 1:1:2 yields the rotaxane **6** directly in 11 per cent yield.

Parallel studies have been carried out by the Vögtle group⁷ as well as two others at the University of Sheffield⁸ and the University of Manchester Institute of Science and Technology⁹. They have each employed host-guest chemistry related to that just described to produce new examples of amide-containing [2]-catenanes — molecules incorporating two rings that are mechanically interlocked like the links of a chain.

The rapid development of such 'molecular engineering' procedures is providing a new focus for chemistry, the fruits of which are already underpinning the quest by chemists to synthesize molecular-scale devices. The latter include systems for information storage, energy conversion, and small-molecule and ion sensing, as well as for use as nanoscale electronic components such as switches, wires and junction boxes. Although the timescale for achieving useful molecular devices remains uncertain, the impressive progress made so far seems set to continue. □

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Conformational change

Jonathon Pines

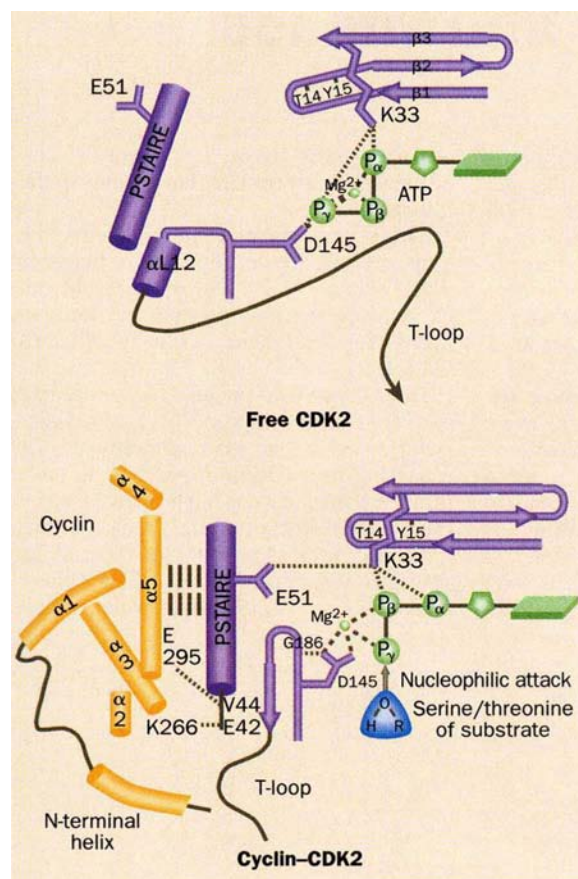
It is not often that nature does as one predicts. When the inactive, monomeric form of CDK2 (cyclin-dependent kinase 2) was solved in 1993, the conformational changes that cyclin binding would induce in order to activate the kinase were predicted¹. Now the crystal structure of a partially active cyclin A-CDK2 complex, solved by Jeffrey *et al.* and described on page 313 of this issue², spectacularly con-

firmes these predictions. The cyclin-CDK complex is activated fully by the phosphorylation of a threonine residue in the T-loop of the CDK by a specific CDK-activating kinase (CAK). The cyclin-CDK complex can then be held in check by phosphorylation on a threonine and/or tyrosine residue in the ATP-binding site of the CDK, or by binding to one of a number of CDK inhibitor proteins (reviewed in ref. 3).

This multifaceted regulation has made the CDK family a prime subject for crystallographic studies.

The first step in providing a molecular basis for how the CDKs are regulated came with the solution of the structure of monomeric CDK2 (ref. 1). From a comparison with the active protein kinase A (PKA) structure⁴, two reasons for the inactivity of CDK2 were immediately apparent. First, ATP is bound in a conformation that would prevent a nucleophilic attack on the scissile β - γ phosphate bond by the substrate hydroxyl group. Second, the 'T-loop' domain blocks the catalytic cleft between the amino- and carboxy-terminal lobes. The main reason for the differences in structure between CDK2 and PKA appeared to be an α -helical region (α L12) unique to CDK2 that constrains both the ATP-binding site and the T-loop domain. Thus cyclin binding was predicted to melt the α L12 helix, allowing the ATP-binding site to reconfigure to resemble that of PKA and enable the T-loop to move away from the catalytic cleft¹.

The second step in the molecular dissection of



The conformational change in the active site of CDK2 induced by cyclin binding. Cyclin alters the orientation of the PSTAIRE helix to bring Glu 51 into the correct position to interact with Lys 33 and Asp 145. These three residues together bring the β - γ bond of ATP into alignment with the substrate hydroxyl group. Cyclin also causes the T-loop to move out of the way of the catalytic cleft. (Adapted from ref. 16.)

firmes these predictions. Moreover, the structure reveals an aspect of cyclin structure that may point to similarities with proteins of the retinoblastoma family and the transcription factor TFIIB.

The cell cycle is regulated by the sequential activation of the cyclin-dependent kinases (CDK) by their partner cyclins. Cyclin-CDK complexes are implicated in the initiation of both DNA replication and mitosis. Monomeric CDKs are inactive as protein kinases and absolutely depend on binding a cyclin for

CDK regulation has been achieved with the solution of a partially active cyclin-CDK complex. Jeffrey *et al.*² have solved the structure of CDK2 bound to an amino-terminal truncated form of cyclin A. This complex is only partially active because the T-loop threonine, Thr 160, is not phosphorylated. Gratifyingly, the crystal structure of this cyclin A-CDK2 complex has borne out most of the predictions made from the monomeric CDK2 structure. ATP is indeed bound in the same manner as in PKA, the T-loop has moved

- Breidenbach, S., Ohren, S., Nieger, M. & Vögtle, F. *J. chem. Soc., chem. Commun.* 1237–1238 (1995).
- Vögtle, F. *et al. Justus Liebigs Ann. Chem.* 739–743 (1995).
- Josten, W. *et al. Chem. Ber.* **127**, 767–777 (1994).
- Kohnke, F. H., Mathias, J. P. & Stoddart, J. F. *Topics curr. Chem.* **165**, 3–49 (1993).
- Lokey, R. S. & Iverson, B. L. *Nature* **375**, 303–305 (1995).
- Hunter, C. A. *J. chem. Soc., chem. Commun.* 749–751 (1991).
- Ottens-Hildebrandt, S. *et al. J. chem. Soc., chem. Commun.* 777–778 (1995).
- Hunter, C. A. *J. Am. chem. Soc.* **114**, 5303–5311 (1992).
- Johnston, A. G., Leigh, D. A., Pritchard, R. J. & Deegan, M. D. *Angew. Chem. Int. Edn. engl.* **34**, 1209–1212 (1995).

away from the catalytic cleft, and the cleft itself has opened up, allowing substrates to bind. Furthermore, Thr 160 is now much more accessible to phosphorylation by CAK, explaining why CAK only phosphorylates CDKs complexed with a cyclin. The inhibitory sites of phosphorylation on the CDK, Thr 14 and Tyr 15, have moved further into the catalytic cleft in the cyclin-CDK complex, which would enhance their ability when phosphorylated to hinder phosphate transfer from ATP to a bound substrate.

How does cyclin achieve these changes when it binds to a CDK? Alanine scanning and point-mutation analysis had indicated that the critical CDK regions involved are around the T-loop, as well as a highly conserved region of 16 amino acids called PSTAIRE^{5,6}. The PSTAIRE region is also important in determining the specificity of which cyclin binds to which CDK. The cyclin A-CDK2 crystal structure shows that cyclin binds primarily to the amino-terminal lobe of CDK, and, in particular, that two cyclin α -helices clamp to the middle of the PSTAIRE helix by hydrophobic interactions, and to either end of PSTAIRE by hydrogen bonds. This has several effects — it rotates the PSTAIRE helix through about 90°, moving it into the catalytic site; it changes the packing of the amino-terminal lobe; and it melts the α L12 helix. Most importantly, the reorientation of the PSTAIRE helix brings the side chain of the catalytic residue Glu 51 into the active site. In this way the ATP-binding site is reconfigured, and the β - γ phosphate bond of ATP moved into a position favourable for attack from a bound substrate (see figure).

Cyclin also strongly interacts with the amino-terminal portion of the T-loop, which, allied with the melting of the α L12 helix, causes the T-loop to move away from the active site and take up a similar, though not identical, position to that seen for this region in PKA. As already mentioned, Thr 160 is phosphorylated in the fully active kinase and this would stabilize the T-loop in this position through ionic

Cultural developments

LICHENS, such as *Usnea articulata* seen festooning this tree in Saudi Arabia, are partnerships between fungi and algae. The fungi concerned are potential sources of novel, pharmacologically active compounds, but the problem is getting at them: lichen-forming fungi are reputedly hard to culture on their own, away from their algal partner. However P. D. Crittenden *et al.* have managed to isolate an impressive 431 lichen-forming fungi from no fewer than 1,021 phylogenetically diverse species, a success rate of 42 per cent (*New Phytologist* 130, 267–297; 1995). Their survey included notoriously tough subjects such as the so-called 'lichenicolous' fungi which, although not symbionts, are obligate inhabitants of lichens; lichen-forming fungi with cyanobacterial (as opposed to simply algal) partners; and fungi of the order Peltigerales, a group of exclusively lichen-forming fungi thought to be phylogenetically ancient, and therefore hard to tease from their symbionts. The most promising subjects were lichens of shrubby habit,



such as *U. articulata* (although this species was one of the 590 in which the fungal symbiont remained obdurately faithful to its partner). H.G.

interactions with a basic patch of residues in the carboxy-terminal lobe of the protein, which may alter the conformation of the T-loop to resemble more exactly that seen in PKA. Phosphorylation of Thr 160 also seems to stabilize the cyclin-CDK complex itself, so there may also be ionic interactions with the cyclin.

This brings us to the structure of cyclin itself, and here there is quite a surprise. The region of cyclin in this crystal structure is an amino-terminal helix followed by a repeat of two sets of five α -helices. Each repeat consists of a three-helix bundle with the other two helices packed against the side, and the two sets of α -helices can be superimposed on one another. One repeat of α -helices is formed by the 'cyclin box', the 100-amino-acid region that is most conserved between cyclins and had been shown by mutational analysis to be essential for binding to CDKs. The second repeat extends from the end of the cyclin box to about 40 amino acids from the carboxy terminus of the protein, yet it bears only 12 per cent sequence identity with the cyclin box. Interestingly, this repeated structure was first noted by Gibson *et al.*, who also pointed out a similar repeat in TFIIB and the retinoblastoma family of proteins⁷. When the crystal structures of these proteins are solved it will be fascinating to discover whether this is a conserved structural motif.

The structure of the cyclin box means a variety of observations now make sense. Of the five most conserved residues^{8,9} (which have also been shown to be important for CDK binding), Arg 211 and Asp 240 form a buried salt bridge that stabilizes

the interaction between the α 1 and α 2 helices. Two others, Lys 266 and Glu 295, hydrogen-bond with the PSTAIRE region of CDK2 and with each other. The presence of the second repeat extending close to the carboxy terminus of the cyclin may explain why even small deletions from this terminus disable cyclin binding to a CDK. Similarly, deletions extending into the helix amino-terminal to the cyclin box prevent CDK binding^{8,10}, and this helix wraps around both α -helical repeats, stabilizing the buried salt bridge and interacting with the carboxy-terminal lobe of CDK2. The extensive interdigitation of cyclin A and CDK2 also makes it possible that cyclins contribute to the substrate specificity of the cyclin-CDK complex.

However, all is not yet solved. Apart from the unanswered effect of Thr 160 phosphorylation, this crystal structure is not of a full-length cyclin. A fundamental characteristic of cyclin A is its rapid degradation in mitosis by an ubiquitin-dependent pathway. The region recognized by the ubiquitination machinery is the 'destruction box'¹¹, which is in the amino-terminal part of the cyclin removed in this crystal structure. Nor does the structure tell us what the cyclin looks like before it binds to the CDK. If cyclin changes its conformation upon binding a CDK, this may be important for interactions with other proteins, such as the CDK inhibitors p21 and p27, and indeed components of the cyclin destruction machinery^{9,12–15}. □

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1. De Bondt, H. L. *et al.* *Nature* **363**, 595–602 (1993).
2. Jeffrey, P. D. *et al.* *Nature* **376**, 313–320 (1995).
3. Morgan, D. O. *Nature* **374**, 131–134 (1995).
4. Knighton, D. R. *et al.* *Science* **253**, 407–414 (1991).
5. Ducommun, B., Brambilla, P. & Draetta, G. *Molec. cell. Biol.* **11**, 6177–6184 (1991).
6. Endicott, J. A., Nurse, P. & Johnson, L. N. *Protein Engng* **7**, 243–253 (1994).
7. Gibson, T. J., Thompson, J. D., Blocker, A. & Kouzarides, T. *Nucleic Acids Res.* **22**, 946–952 (1994).
8. Kobayashi, H. *et al.* *Molec. Biol. Cell* **3**, 1279–1294 (1992).
9. Stewart, E., Kobayashi, H., Harrison, D. & Hunt, T. *EMBO J.* **13**, 584–594 (1994).
10. Lees, E. M. & Harlow, E. *Molec. cell. Biol.* **13**, 1194–1201 (1993).
11. Glotzer, M., Murray, A. W. & Kirschner, M. W. *Nature* **349**, 132–138 (1991).
12. Herskko, A. *et al.* *J. Biol. Chem.* **269**, 4940–4946 (1994).
13. Irriger, S. *et al.* *Cell* **81**, 269–277 (1995).
14. King, R. W. *et al.* *Cell* **81**, 279–288 (1995).
15. Tugendreich, S. *et al.* *Cell* **81**, 261–268 (1995).
16. Pines, J. *Curr. Biol.* **3**, 544–547 (1993).

Resetting the circadian cycle

Michael Hastings

THESE are exciting times in chronobiology. Since their identification 40 years ago, we have known that our circadian clocks have an intrinsic period of about 25 solar hours. They therefore need frequent resetting by the environment (that is, entrainment) if they are to be effective: however, as we knew nothing of the basic biochemical mechanisms that constitute a clock, we could not start to explain how one might be reset. Writing in *Cell*, Crosthwaite *et al.*¹ now describe how the molecular cycle of the circadian clock in the fungus *Neurospora* is brought into synchrony with the cycle of light and dark. Their paper is a landmark in our understanding of circadian clockwork, and provides an elegant biochemical explanation of those formal properties of entrainment that are common to all species, including ourselves.

When held in constant twilight, circadian clocks define the organism's subjective day and night, free-running at their intrinsic period. They can be reset by brief pulses of light which mimic dawn and dusk, the critical observation being that the direction and magnitude of the phase shift depend on when the light falls². The phase-response curve of the *Neurospora* clock shows delays to light encountered in subjective afternoon and evening, and advances during late subjective night and morning³. This raises two questions: what is the biochemical oscillation at the heart of the clock, and how does it respond to light in this complex way?

One current model of the clock is based on autoregulatory loops in which the protein products of 'clock genes' such as *per* in *Drosophila* and *frq* in *Neurospora* enter the nucleus after a post-transcriptional lag to inhibit further expression (see figure). Transcription only restarts when inactivation of the negative feedback, possibly by degradation of the 'clock protein' or its dependent factors, releases the clock gene^{4,5}. Many transcriptional pathways are probably oscillatory, but the defining feature of the clock is that the elements mesh together in a self-sustaining cycle of approximately one

solar day. This model holds that the clock proteins of fungus and fly are central clock components whose instantaneous abundance and whose rate and direction of change establish circadian time.

Crosthwaite *et al.*¹ advance this model significantly by exploring how light affects transcription of *frq*, and having done so, they are able to explain circadian entrainment as a molecular dynamic. They dem-

onstrate that a brief light pulse overrides any feedback and causes a large and rapid induction of *frq*, which correlates directly with the size of the clock's anticipated phase shift. Consistent with the critical role of the putative protein FRQ in pacing the transcriptional cycle, when they inhibited *de novo* protein synthesis, they also blocked circadian resetting by light, even though *frq* was still induced, showing that you cannot reset the clock without producing extra (FRQ?) protein.

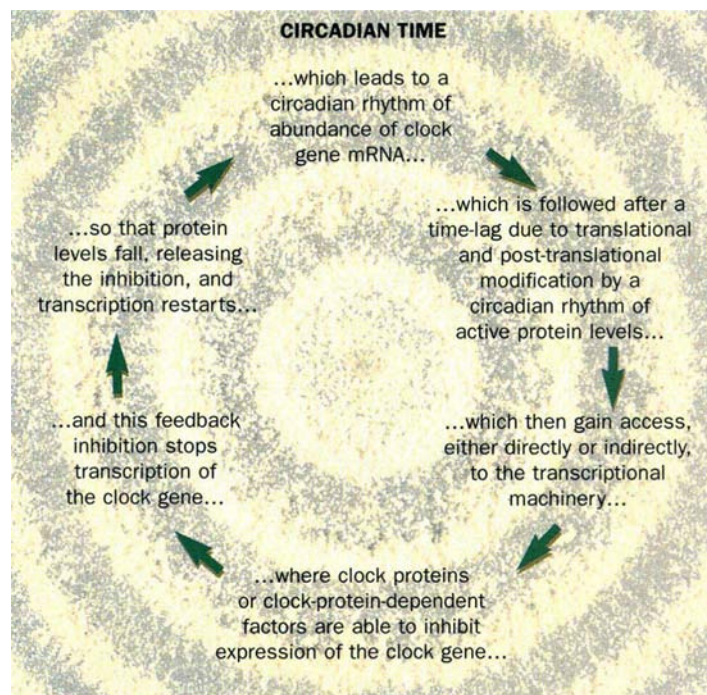
So how is this related to the autoregulatory cycle and the phase-response curve? Well, during late subjective day and evening, spontaneous transcription of *frq* and, by inference, levels of FRQ are falling. If

exposure to light acutely induces transcription, the model predicts that the spontaneous decline should be slowed, and there would be a temporary increase in protein levels. Consequently, escape of the gene from inhibitory feedback and the start of the next clock cycle would be delayed. Conversely, transcription of *frq* is just starting during subjective night, and induction by light would accelerate the rise and thereby advance the cycle and the fungal rhythms it drives — exactly as we see in the phase-response curve. These findings also explain another long-standing observation that constant light makes *Neurospora* arrhythmic because sustained induction of *frq* would lock FRQ at high levels, so stopping the clock. In general outline then, not only does the autoregulatory cycle of *frq* describe the spontaneous circadian period, but when combined with the unidirectional effect of light on *frq* transcription, it also provides a molecular explanation of the bidirectional phase-response curve. A simple response gives rise to an apparently complex behaviour.

How universal is this model of the clock? We definitely need to know more of the properties of the putative product FRQ because the model holds that this, or FRQ-dependent factors, regulates the transcriptional machinery and propels the loop. In *Drosophila* the properties and cellular distribution of PER, the product of the clock gene *per*, are better understood and are consistent with the model, although PER is not independently active as a transcription factor, and its role in resetting the clock by

light is still to be determined. By analogy with the *frq* story, the temporal relationship between the transcriptional cycle and the phase-response curve of the fly is best explained by light suppressing rather than stimulating transcription of *per*, and there are some suggestions that this does happen.

The products of mammalian clock genes are even less well understood, but it is clear that the phase-response curve of many species incorporates a large dead-zone during subjective day when light is ineffective. This is consistent with an inhibitory effect of light on transcription of clock genes but might also reflect a circadian gating of the phototransduction



Photograph of *Neurospora* by P. Lakin-Thomas

Circadian clocks: a never-ending sentence. Circadian rhythms of transcription of clock genes lead to increased abundance of mRNA, which is followed by peak availability of clock proteins some hours later. The proteins are able, directly or indirectly and through unknown mechanisms, to inhibit transcription of their encoding gene. This transcriptional inhibition cannot be sustained because in the absence of transcription, levels of clock protein will inevitably decline; transcription will therefore restart and a self-sustaining oscillation is generated. The stable circadian period is presumed to arise from kinetics of the constituent events, for example the rates of synthesis, modification and degradation of mRNA and protein, and rates of transfer between cytoplasm and nucleus.

onstrate that a brief light pulse overrides any feedback and causes a large and rapid induction of *frq*, which correlates directly with the size of the clock's anticipated phase shift. Consistent with the critical role of the putative protein FRQ in pacing the transcriptional cycle, when they inhibited *de novo* protein synthesis, they also blocked circadian resetting by light, even though *frq* was still induced, showing that you cannot reset the clock without producing extra (FRQ?) protein.

So how is this related to the autoregulatory cycle and the phase-response curve? Well, during late subjective day and evening, spontaneous transcription of *frq* and, by inference, levels of FRQ are falling. If

pathway, temporarily rendering the clock 'blind'. Moreover, it is clear that the mammalian clock is sensitive both to light and to non-photic cues associated with arousal. The phase response curves to these stimuli are very different⁶, making it difficult to accommodate them in a single molecular oscillation. It is more likely that a number of intermeshing molecular cycles, addressed selectively by particular cues, sustain clock function in most, if not all, species. Encouragement that these cycles may be identified in mammals comes from new findings by Welsh *et al.*⁷ that isolated neurons of the suprachiasmatic nucleus, the site of the mammalian clock, can express independent circadian rhythms. This indicates that the mammalian clock may well be cell-autonomous, a

prerequisite of an autoregulatory cycle. The pivotal role of *frq* in generating and resetting circadian time provides an excellent template for further studies seeking to unravel these loops. □

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1. Crosthwaite, S. K., Loros, J. J. & Dunlap, J. C. *Cell* **81**, 1003–1012 (1995).
2. Daan, S. & Pittendrigh, C. S. *J. comp. Physiol.* **A106**, 255–266 (1976).
3. Sargent, M. L. & Briggs, W. R. *Plant Physiol.* **42**, 1504–1510 (1967).
4. Aronson, B. D. *et al. Science* **263**, 1578–1584 (1994).
5. Edery, I., Hardin, J. E. & Rosbash, M. *Science* **263**, 237–240 (1994).
6. Smith, R. D., Turek, F. W. & Takahashi, J. S. *Am. J. Physiol.* **262**, R1149–R1153 (1992).
7. Welsh, D. K. *et al. Neuron* **14**, 697–706 (1995).

OZONE DEPLETION

CFC alternatives under a cloud

Steven E. Schwarzbach

To reduce the rates of depletion of stratospheric ozone, the international community has agreed to accelerate restrictions on the use of chlorofluorocarbons (CFCs)¹. This accelerated phase-out depends, of course, on the manufacture of replacements; candidate replacement compounds (see table) include seven hydrochlorofluorocarbons (HCFCs) and five hydrofluorocarbons (HFCs). A rapid phase-out of CFCs has necessarily meant rapid evaluation of the attendant environmental risks of the twelve replacement compounds and their potential degradation products. The scientific consensus has been that HFCs and HCFCs are clearly environmentally preferable from the point of view of reducing ozone depletion and they have been hopefully and aggressively pursued by industry as the logical successors to CFCs. But on page 327 of this issue Tromp and co-authors² reveal a potential new risk: accumulation of trifluoroacetic acid, a breakdown product of three of the proposed CFC replacements, in certain wetlands.

Trifluoroacetic acid (TFA) is produced in the atmosphere through interaction of OH radicals with three CFC replacement compounds: HCFC-123 and HCFC-124 (CHCl_2CF_3 and CHClFCF_3) and HFC-134a (CH_2FCF_3). The latter compound is expected to be one of the most important CFC replacements for refrigeration and air-conditioning purposes between now and the year 2025. But it will also be the dominant TFA precursor, with annual production expected³ to approach 300 thousand tonnes by 2025. TFA is resistant to abiotic degradation processes such as photolysis and hydrolysis and is virtually unmetabolizable by most plants and animals⁴. Experimental systems designed

to evaluate the potential for microbial degradation of TFA have had some success, yet it is far from clear how much degradation can be expected in nature⁵. Where degradation by bacteria has been achieved it has ceased at concentrations greater than $100 \mu\text{g l}^{-1}$.

Within a matter of days after entering the atmosphere, TFA is expected to distribute from air to cloud water, whence it will return to the Earth's surface via precipitation. By the year 2010 global emissions are projected to result in an average global TFA concentration of $0.160 \mu\text{g}$ per litre of precipitation. This average concentration is three orders of magnitude below toxic thresholds of the most sensitive species yet assessed, and in the models of Tromp *et al.* these concentrations do not result in significant accumulations in wetlands, even without biodegradation.

The problem is that there are a variety of factors capable of enhancing the local source strength of TFA. Tromp *et al.* note that TFA concentrations in precipitation of arid and semi-arid regions may be 2–4 times greater than the global mean. The

atmospheric source strength of TFA may be further and substantially enhanced in urban areas because of high densities of source emitters. As evidence, Tromp *et al.* cite measurements of CFCs in urban areas over the past 20 years which have been found to be up to 10 to 20 times the zonal averages. In addition, urban areas present another enrichment factor — air pollution. The enrichment of urban air with $\text{OH}^\bullet$ levels as much as 10 times the zonal mean can lead to another order-of-magnitude increase by increasing the rate of TFA formation. Local TFA concentrations in precipitation of 2 to $20 \mu\text{g l}^{-1}$ or more suddenly appear plausible.

Water bodies characterized by little to no outflow and high evaporation rates may be particularly susceptible to accumulating rain-borne TFA. Surface water concentrations may be further enhanced in these wetlands by an order of magnitude or more⁶. Ultimately, the TFA concentrations to be expected in wetlands will depend on TFA source strength, enhancement factors and loss. In hydrologically and topographically closed wetlands such as playa lakes, or partially closed wetlands such as vernal pools, loss will be limited to minor seepage, aeolian losses and biodegradation.

Tromp *et al.* construct two models for these types of wetlands. Their first model predicts that if seepage and aeolian transport losses from a wetland are limited to 1 per cent per year, a concentration of $100 \mu\text{g l}^{-1}$ can be achieved in 50 years, given a source concentration between 2 and $3 \mu\text{g l}^{-1}$. In their second model Tromp *et al.* develop a concentration-dependent biodegradation equation using previously reported data⁵ and estimate that with rainfall concentrations of only $1 \mu\text{g l}^{-1}$ and an enhancement factor of fivefold, concentrations of $100 \mu\text{g l}^{-1}$ could be achieved in as few as 30 years, even where seepage is as much as 10 per cent.

Wetlands that tend to concentrate conservative solutes are diverse and exist on every continent. They include saline sinks, vernal pools, playa lakes, aestival ponds (ponds that freeze to the bottom for two to eight months a year) and temporary pools in bedrock depressions. The extent and significance of these wetlands is sub-

Potential alternatives to CFCs

Hydrochlorofluorocarbons		Hydrofluorocarbons	
HCFC-22	CHClF_2	HFC-32	CH_2F_2
HCFC-123	CHCl_2CF_3	HFC-125	CHF_2CF_3
HCFC-124	CHClFCF_3	HFC-134a	CH_2FCF_3
HCFC-141b	$\text{CH}_3\text{Cl}_2\text{F}$	HFC-143a	CH_3CF_3
HCFC-142b	CH_3CClF_2	HFC-152a	CH_3CHF_2
HCFC-225ca	$\text{CHCl}_2\text{CF}_2\text{CF}_3$		
HCFC-225cb	$\text{CHClFCF}_2\text{CClF}_2$		

Compounds in red produce TFA in the atmosphere.



Vernal pools are seasonal wetlands, collecting water in the winter rainy season and drying out during the summer drought which is typical of Mediterranean climates. They may be particularly susceptible to high concentrations of TFA, if those concentrations occur.

stantial. The volume of saline lake water alone, $104,000 \text{ km}^3$, is almost as great as the volume of the world's fresh waters, $125,000 \text{ km}^3$. Arid land wetlands are also unique biogeographically, typically functioning as habitat islands in a sea of land. These wetlands are consequently often refuges for remnant endemic species, such as desert pup fish, fairy shrimp or rare plants, and a variety of other, often ecologically vulnerable, species.

Enhancement of solute concentrations is not limited to surface wetlands alone. Shallow groundwater in arid regions may also be solute-enhanced by evaporation. Evaporative concentration factors as high as 170-fold have been measured for perched groundwater in the Imperial Valley of southern California⁷.

The effects of TFA on the Earth's biota, if any occur, are likely to be first observed in plants. TFA is highly mobile in xylem tissue and has been demonstrated to bioaccumulate by at least a factor of 30 in vascular plants⁸. In late 1993 and early 1994, TFA was detected in the atmosphere near Tübingen, Germany (at parts-per-trillion concentrations) and in spruce needles in Sweden (at parts-per-billion concentrations)⁹. The source of current environmental TFA is thought to be the atmospheric degradation of the anaesthetic halothane, a compound with comparatively minor annual emissions. Given the high ratio of rooted vascular plant biomass to water volume in the vernal pools of southern California, their proximity to the Los Angeles Basin airshed and the arid climate of this region, it seems possible that plants in these wetlands might ultimately accumulate TFA to concentrations in the parts-per-million range.

As of now, we have no idea what tissue

concentrations in plants are harmful. Future areas of research, in my view, must focus on the potential of TFA to accumulate to toxic levels in the Earth's most susceptible wetlands and vegetation. Tissue toxicity levels and bioaccumulation factors for TFA in a wide variety of species must continue to be pursued. More information is also needed on the robustness of degradation pathways in different wetland settings. Sufficiently sensitive analytical methods should be developed and published, making it easier for investigators worldwide to track current concentrations of TFA in the environment, while concentrations remain toxicologically insignificant.

Clearly a transition away from CFCs must be made. As sometimes happens, however, the solution to one problem is not without unintended risks. TFA is likely to be an extremely persistent and globally distributed compound — but will it be universally benign when produced and released in kilotonne quantities? □

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1. Parson, E. A. & Greene, O. *Environment* **37**, 16–43 (1995).
2. Tromp, T. K., Ko, M. K. W., Rodriguez, J. M. & Sze, N. D. *Nature* **376**, 327–330 (1995).
3. McCulloch, A. *Envir. Monit. Assessmt* **31**, 167–174 (1994).
4. United Nations Environment Programme *Environmental Effects of Ozone Depletion: 1994 Assessment* (UNEP, Nairobi, November 1994).
5. Visscher, P. T., Culbertson, C. W. & Oremland, R. S. *Nature* **369**, 729–731 (1994).
6. Sefchick, J., Skorupa, J. & Schwarzbach, S. *AFEAS Rep. No. CTR91-18*, 16 (1994).
7. Saiki, M. K. *Water Air Soil Pollut.* **52**, 41–56 (1990).
8. Thompson, R., Stewart, K. M. & Gillings, E. *AFEAS Rep. No. SP91-18*, 8 (1994).
9. Frank, H. *AFEAS Workshop Environmental Fate of Trifluoroacetic Acid* (Miami Beach, 3–4 March, 1994).

DAEDALUS

Washing the car

THE exhaust fumes from internal-combustion engines are increasingly resented, especially in cities. Yet they seem unavoidable. A flame in a closed cylinder is inevitably quenched on the cold surfaces, giving incomplete combustion. Daedalus now has a new approach.

Imagine, he says, a porous engine, in which air is pumped continuously into the cylinders through their porous walls. The flame would be swept back, and would never touch the cylinder walls; internal combustion would go completely to carbon dioxide and steam. The major problem is lubrication. Oil on the metal surfaces would block the pores, and act as a cold quenching surface for the flame. At first Daedalus hoped that the air itself would serve as a lubricant. Air bearings, in which compressed air emerges from many small holes in one surface, are widely used in ruling engines and low-friction mechanisms. But air is too compressible, and has too low a viscosity, to lubricate the violently and intermittently loaded bearing surfaces of a car engine. Very high pressures would be needed to stop the surfaces from touching; the resulting air flow would exceed the exhaust capacity of the engine.

So Daedalus will lubricate his porous engine not with air, but with a safely incompressible liquid: water. While being pumped through the pores of the hot structure, the water will boil. The bearings of the engine will be splendidly lubricated by boiling, expanding, steam-pressurized water, and its cylinder walls will exude steam. As well as keeping the flame away from these surfaces, the steam will 'crack' any incompletely reacted fuel to carbon monoxide and hydrogen, which will burn to completion. The expanding steam may even usefully augment the power of the engine, in effect capturing some of its waste heat.

Daedalus will make the porous bearing surfaces of his engine, not of metal, but of advanced ceramics. Ceramics resist thermal shock; many of them are inherently porous, and ceramic, uncooled 'adiabatic' diesel engines have already been tested for military use. Daedalus's water-cooled engine will be a far less demanding application.

The new engine should give motoring a welcome ecological respectability. Its clean exhaust, freedom from dirty polluting oil and lack of a radiator to boil, freeze or leak, should fully compensate for the need to fill up with water as well as fuel at every stop. It will also give journalists the chance to air once more that old canard of 'a car which runs on water'.

David Jones

Making a meal of mother

SIR — All spiders provide some form of maternal care, from weaving protective silk around the eggs to guarding and feeding spiderlings¹. An unusual and extreme form of care is matrophagy, where the spiderlings consume their mother. Although widely reported¹⁻³, the significance of this behaviour has not been explored. Here we describe a novel form of maternal care that includes matrophagy in the Australian social spider *Diaea ergandros* Evans (Thomisidae).

Mothers store food in unviable trophic eggs that are never laid. The nutrients stored in these eggs appear to be converted to haemolymph which is consumed by 'nursing' spiderlings. Later, the partially depleted eggs are consumed directly when the offspring eat their mother's entire body. Mothers that store more food support longer periods of matrophagous feeding, thereby reducing sibling cannibalism.

Maternal care is more extensive among social than other spiders⁴⁻⁶. *D. ergandros* mothers build a large, protective nest with eucalyptus leaves, and capture large insect prey to feed their brood. The survival of spiderling *D. ergandros* depends on the mother's presence until they reach their fourth instar, after which they are capable of foraging and nest construction^{7,8}.

We examined the ovaries of female *D. ergandros* at different reproductive stages (Fig. 1). Gravid females had elongated ovaries containing many small oocytes. Female *D. ergandros* lay only a single clutch, unlike other thomisids³, and after oviposition their ovaries become shrivelled, with no oocytes. However, mothers regain weight after oviposition and have unusual ovaries that contain significantly fewer ($t = 4.22$, $P < 0.01$) and larger ($t = 20.15$, $P < 0.001$) oocytes than those of gravid mothers. Histological examination revealed that the oocytes in the redevel-

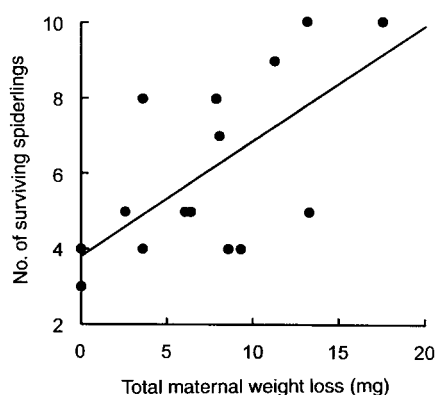


FIG. 2 The number of juveniles surviving the starvation period was positively correlated with the weight lost by mothers ($r = 0.65$, $F_{1,15} = 9.60$, $P < 0.01$, $n = 16$), but not total spiderling weight ($r = 0.281$, $F_{1,15} = 1.119$; not significant).

oped ovaries were distorted and appeared to be nonviable; yolky granules filled the cytoplasm and no nuclear material had been stained.

Spiderlings do not obtain the nutrients contained in these oocytes by simply eating them because, unlike trophic eggs⁹, the oocytes are larger than the oviducts and cannot be laid. Instead, the fourth-instar spiderlings acquire these nutrients when they slowly cannibalize their mother. Initially, they imbibe small quantities of haemolymph from the leg joints of the living, unresisting mother. As matrophagy continues, the mother loses mobility and her opisthosoma shrinks, presumably as the trophic eggs are transformed into haemolymph. After several weeks, she is decrepit, unable to move, and the offspring eat her entirely.

The growth and survival of mothers and spiderlings was examined for 6 weeks. The brood gained weight (6.5 ± 2.8 mg, $n = 16$) during the first 3 weeks of the

starvation period, which corresponded with the weight lost by their mothers (8.0 ± 1.7 mg, $n = 16$). The weight lost by the mother during the starvation period affected the duration of matrophagy and hence juvenile survival. Lighter mothers were entirely consumed earlier in the experiment than heavier mothers, and thus cannibalism among the spiderlings of these lighter, deceased mothers occurred earlier. Consequently, the number of surviving offspring at the end of the experiment was positively correlated with the weight lost by the mothers over the starvation period (Fig. 2).

Extreme forms of parental care, such as matrophagy, may be most likely among spiders that typically produce single clutches. Additionally, matrophagy may facilitate the evolution of social behaviour⁹ by reducing cannibalism among groups of siblings^{10,11}, as occurs in *D. ergandros*⁷ and other social species^{6,11}.

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1. Foelix, R.F. *Biology of Spiders* (Harvard Univ. Press, Cambridge, 1982).
2. Barnes, J. *The Complete Works of Aristotle* (Princeton Univ. Press, 1984).
3. Bristowe, W. S. *The World of Spiders* (Collins, London, 1958).
4. D'Andrea, M. *Monitore zool. ital. NS Monogr.* 3 (1987).
5. Buskirk, R. E. in *Social Insects* (ed. Hermann, H. R.) 281-387 (Academic, London, 1981).
6. Selbt, U. & Winkler, W. *Anim. Behav.* 35, 1903-1905 (1987).
7. Evans, T. A. *Rec. W. Aust. Mus. (Suppl.)* 51, 151-158 (1995).
8. Main, B. Y. in *Australian Arachnology* (eds Austin, A. D. & Heather, N. W.) 55-73 (Aust. Ent. Soc., Brisbane, 1988).
9. Crespi, B. J. in *Cannibalism: Ecology and Evolution among Diverse Taxa* (eds Elgar, M. A. & Crespi, B. J.) 176-213 (Oxford Univ. Press, 1992).
10. Alexander, R. D. A. *Rev. ecol. Syst.* 5, 325-383 (1974).
11. Elgar, M. A. & Crespi, B. J. (eds) in *Cannibalism: Ecology and Evolution among Diverse Taxa* 1-12 (Oxford Univ. Press, 1992).

Opening up Ca^{2+} stores with InsP_3

SIR — Hirose and Iino¹ introduced the low-affinity Ca^{2+} -sensitive dye Fura2 into intracellular Ca^{2+} stores of vascular smooth-muscle cells and measured the kinetics of inositol trisphosphate (InsP_3)-induced Ca^{2+} release. This novel technique of incubating intact cells with Ca^{2+} buffers like Fura2/AM (ref. 1) or Fura-2/AM (refs 2-5) to load them in the intracellular stores, followed by permeabilization of the plasma membrane, allows fast measurements of the luminal Ca^{2+} concentration ($[\text{Ca}^{2+}]$) in the presence of a constant cytosolic $[\text{Ca}^{2+}]$. As luminal Ca^{2+} can control InsP_3 -induced Ca^{2+} release⁶, we investigated in

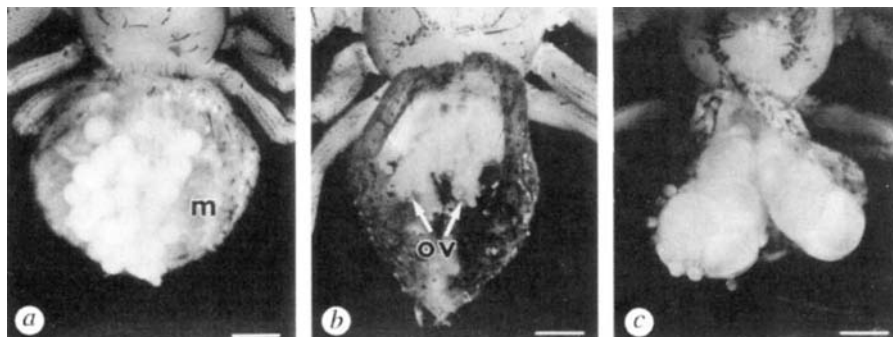
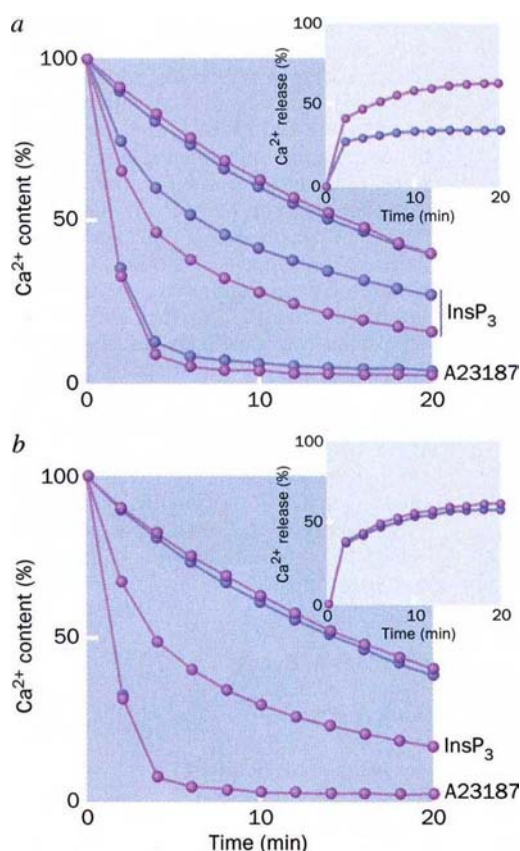


FIG. 1 a, A gravid female (44.54 ± 2.05 mg, $n = 22$), with the left ovary exposed, surrounded by midgut diverticula (m). Ovaries contained 41 ± 6.02 oocytes ($n = 8$) 0.26 ± 0.01 mm in diameter. b, A female one week after oviposition (22.50 ± 0.54 mg, $n = 22$) with a completely eviscerated opisthosoma; ovaries (ov) extremely small, without oocytes ($n = 7$). c, A mother one month after oviposition (47.13 ± 1.69 mg, $n = 22$) with enlarged, irregular ovaries. Ovaries contained 13 ± 1.51 oocytes ($n = 8$) 0.77 ± 0.03 mm in diameter. Scale bars, 1 mm.



A7r5 smooth muscle cells whether the presence of a Ca^{2+} -sensitive dye in the stores would interfere with the kinetics of Ca^{2+} mobilization. InsP_3 at $0.5 \mu\text{M}$ became less effective in emptying stores if the cells were pretreated with $40 \mu\text{M}$ Fura-2/AM for 3.5 h using the protocol of ref. 1 (see Fig. 1a). No similar inhibition occurred if $2 \mu\text{M}$ Fura-2/AM was applied for 1 h or less (see Fig. 1b; protocol as in refs 2, 4). We conclude that high concentrations of low-affinity Ca^{2+} -sensitive dyes in the stores can seriously affect Ca^{2+} release through the InsP_3 receptor.

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FIG. 1 Effect of Fura-2 and Fura-2/AM on InsP_3 -induced Ca^{2+} release. Intact A7r5 cells (pink symbols) were pretreated at 37°C with a, Fura-2/AM ($40 \mu\text{M}$, 3.5 h) and 0.005% Pluronic-F127 or b, Fura-2/AM ($2 \mu\text{M}$, 1 h). Control cells (purple symbols) were pretreated with a, 0.1% DMSO plus 0.005% Pluronic-F127 or b, DMSO alone. The cells were then permeabilized at 25°C as described⁶. Confocal microscopy verified that the dye was present in the lumen of the stores. The stores were loaded with $^{45}\text{Ca}^{2+}$ for 35 min in 120 mM KCl, 30 mM imidazole (pH 6.8), 5 mM MgCl_2 , 5 mM ATP, 0.44 mM EGTA, 10 mM NaN_3 and 100 nM free $^{45}\text{Ca}^{2+}$ and then incubated in 120 mM KCl, 30 mM imidazole (pH 6.8), 2 mM MgCl_2 , 1 mM ATP, 1 mM EGTA, 100 nM free $^{40}\text{Ca}^{2+}$ and $2 \mu\text{M}$ thapsigargin. This medium was replaced every 2 min. The Ca^{2+} contents of the stores during this incubation and the effects of a 20-min stimulation with $0.5 \mu\text{M}$ InsP_3 or $10 \mu\text{M}$ A23187 are shown. The insets give the time course of InsP_3 -induced Ca^{2+} release normalized to the A23187-releasable Ca^{2+} . Values are means of 13 (a) and 5 (b) independent experiments. The standard error of the mean was always less than 2.5%. Pretreatment with Pluronic-F127 did not affect InsP_3 -induced Ca^{2+} release (data not shown).

SIR — Although there has been considerable interest and research over the past few years in the kinetics and regulatory properties of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel (InsP_3 receptor) — including several articles in *Nature*^{1,5-8} — the quantal and biphasic nature of InsP_3 -induced Ca^{2+} release remains an enigma. Many papers espouse one (or both) of the two models proposed for quantal InsP_3 -induced Ca^{2+} release, a phenomenon by which submaximal concentrations of InsP_3 are unable to evoke total Ca^{2+} release from InsP_3 -sensitive stores while the rates of release decline with time until higher concentrations of InsP_3 are added^{1,9}. One model, originally proposed by Irvine¹⁰, suggests that luminal Ca^{2+} mediates this process, so that a fall in the Ca^{2+} content of the InsP_3 -sensitive stores during channel opening serves to close the channel. The other model favours the view that InsP_3 -sensitive Ca^{2+} stores discharge their Ca^{2+} content in an all-or-none manner, with the stores being

heterogeneous in their sensitivities to InsP_3 (ref. 11).

The recent article by Hirose and Iino¹ has gone some way to resolve this issue. By monitoring luminal Ca^{2+} concentrations, they were able to show that biphasic InsP_3 -induced Ca^{2+} release was not significantly affected by luminal Ca^{2+} concentrations. They also showed that the InsP_3 -sensitive stores do not release Ca^{2+} in an all-or-none manner and that the channels do not become inactivated on InsP_3 exposure (directly contradicting the conclusion in ref. 5). Hirose and Iino proposed that biphasic Ca^{2+} release occurs through two populations of InsP_3 -sensitive Ca^{2+} stores, each releasing Ca^{2+} monoexponentially but with different rates (fast and slow), such that the observable extent of Ca^{2+} release with time is biphasic. After initially assuming a biexponential process for Ca^{2+} release, they calculated a single $t_{1/2}$ for Ca^{2+} release curves observed at 30 and 100 nM InsP_3 and showed that both traces had the same shape, although on different scales, as the $10 \mu\text{M}$ InsP_3 Ca^{2+} release trace. From this they concluded that the Ca^{2+} stores must have homogeneous sensitivities to InsP_3 and be heterogeneous in their InsP_3 receptor densities.

A more rigorous approach is to calculate the rate constants for both components of Ca^{2+} release at each InsP_3 concentration ($[\text{InsP}_3]$) and then calculate the ratio of the two rate constants. This ratio should be constant for all $[\text{InsP}_3]$ used if the sensitivities of the stores to

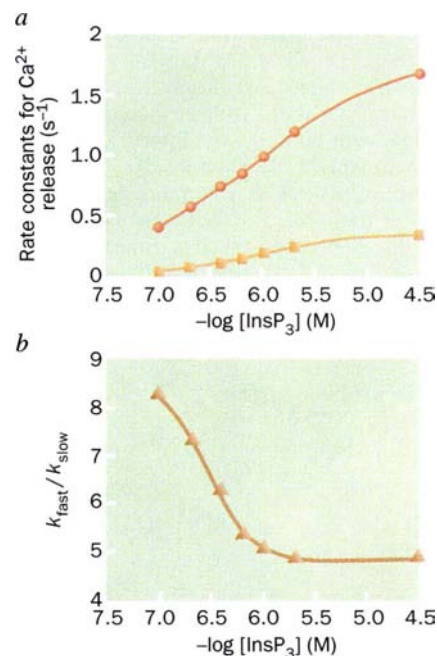


FIG. 2 The rate constants for biphasic InsP_3 -induced Ca^{2+} release using rat cerebellar microsomes. a, InsP_3 -induced Ca^{2+} release was monitored by changes in fluo-3 fluorescence¹³ using a stopped-flow spectrofluorimeter. b, Dependence of the ratio of the two rate constants on $[\text{InsP}_3]$.

1. Hirose, K. & Iino, M. *Nature* **372**, 791–794 (1994).

2. Glennon, M. C. et al. *J. Biol. Chem.* **267**, 25568–25575 (1992).

3. Hofer, A. M. & Machen, T. E. *Proc. natn. Acad. Sci. U.S.A.* **90**, 2598–2602 (1993).

4. Short, A. D., Klein, M. G., Schneider, M. F. & Gill, D. L. *J. Biol. Chem.* **268**, 25887–25893 (1993).

5. Hajnóczky, G. & Thomas, A. P. *Nature* **370**, 474–477 (1994).

6. Missiaen, L., De Smedt, H., Droogmans, G. & Casteels, R. *Nature* **357**, 599–602 (1992).

7. Ferris, C. D., Cameron, A. M., Huganir, R. L. & Snyder, S. H. *Nature* **356**, 350–352 (1992).

8. Berridge, M. J. *Nature* **361**, 315–325 (1993).

9. Muallem, S., Pandolfi, S. J. & Beeker, T. G. *J. Biol. Chem.* **264**, 205–212 (1989).

10. Irvine, R. F. *FEBS Lett.* **263**, 5–9 (1990).

11. Oldershaw, K. A., Nunn, D. L. & Taylor, C. W. *Biochem. J.* **278**, 705–708 (1991).

12. Champell, P. et al. *J. Biol. Chem.* **264**, 17665–17673 (1989).

13. Brown, G. R., Sayers, L. G., Kirk, C. J., Michell, R. H. & Michelangeli, F. *Biochem. J.* **282**, 309–312 (1992).

14. Iino, M. & Endo, M. *Nature* **360**, 76–78 (1992).

15. Iino, M. *J. gen. Physiol.* **95**, 1103–1122 (1990).

16. Bezprozvanny, I., Watras, J. & Ehrlich, B. E. *Nature* **351**, 751–754 (1991).

17. Finch, E. A., Turner, T. J. & Goldin, S. M. *Science* **252**, 443–446 (1991).

InsP₃ are similar. Figure 2 shows fast and slow rate constants calculated for InsP₃-induced Ca²⁺ release using rat cerebellar microsomes. Both rate constants increase with [InsP₃]; however, the ratio of the fast and slow rate constants is not constant but rather increases at low [InsP₃]. This indicates that the stores are heterogeneous in their sensitivities to InsP₃. This result is unlikely to be a consequence of using a different cell system, because other studies on the kinetics of InsP₃-induced Ca²⁺ release using permeabilized hepatocytes also showed biphasic release¹². The ratios of the fast and slow rate constants when calculated from that paper also varied with [InsP₃].

In the light of data given in refs 1 and 5, we must reassess our current models for the complex kinetic behaviour of the InsP₃ receptor. Heterogeneity of InsP₃ sensitivity of the Ca²⁺ stores cannot be ruled out.

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HIROSE AND IINO REPLY — Missiaen *et al.* report that heavy loading of Fura-2/AM to Ca²⁺ stores affected the Ca²⁺ release induced by InsP₃ in permeabilized A7r5 cells. The Ca²⁺ leakage rate (in the absence of InsP₃) was not changed by Fura-2 loading in their experiments, indicating that the driving force for Ca²⁺ efflux was not changed, that is, the free luminal Ca²⁺ concentration was not altered by the dye loading. The observed effect on the InsP₃-induced Ca²⁺ release would then not result from a dependence of InsP₃-induced Ca²⁺ release on luminal Ca²⁺ concentration, but more probably from a nonspecific effect of their loading procedure. If, on the other hand, there had been a decrease in luminal Ca²⁺ concentration which was somehow cancelled by a nonspecific effect of Fura-2 on the Ca²⁺ leakage pathway, then the observed slowing of the Ca²⁺ release rate could be explained by the decreased driving force alone.

In our view, the observations of Missiaen *et al.* do not require a reinterpretation of our previous results¹ for the following reasons: (1) they used cultured cells, whereas we used intact smooth muscle cells, which react differently to dye treatment; and (2) the shape of the time course of Ca²⁺ release seemed little changed after Fura-2 loading in their experiment, indicating that the kinetics of InsP₃-induced Ca²⁺ release was not seriously affected by the Fura-2 effect.

In summary, although Fura-2 may indeed have minor nonspecific effects on Ca²⁺ release mechanisms under certain experimental conditions, the results of Missiaen *et al.* do not offer definitive proof or disproof of previous hypotheses

on the properties of InsP₃-sensitive stores.

Mezna and Michelangeli report the heterogeneity of InsP₃ sensitivity in Ca²⁺ stores of rat cerebellum. We believe that their and our analyses are mathematically equivalent, but that their experiments are subject to a common pitfall. Their results were obtained by measuring Ca²⁺ concentration released to the cytoplasm using Fluo-3 fluorimetry. When cytoplasmic Ca²⁺ concentration is measured using fluorescent Ca²⁺ indicators, the Ca²⁺ concentration around the Ca²⁺ store inevitably changes during Ca²⁺ release. This change in Ca²⁺ concentration is known to seriously affect the kinetics of Ca²⁺ release¹⁴, because the rate of InsP₃-induced Ca²⁺ release is biphasically dependent on the cytoplasmic Ca²⁺ concentration^{15–17}. The extent of feedback modulation of the InsP₃ receptor by Ca²⁺ on the cytoplasmic side depends on the concentration of InsP₃, which affects the

rate of Ca²⁺ concentration change. This is why analyses of InsP₃-induced Ca²⁺ release without cytoplasmic Ca²⁺ buffering, such as reported by Mezna and Michelangeli, do not allow any conclusion about the InsP₃ sensitivities.

In contrast, we buffered the cytoplasmic Ca²⁺ concentration using 10 mM of either EGTA or BAPTA to avoid the Ca²⁺-mediated feedback effect, and found no heterogeneity in the InsP₃ sensitivity¹. Cytoplasmic Ca²⁺ concentration is as important as InsP₃ concentration in the regulation of InsP₃ receptor activity. It is important to take this point into consideration in trying to understand InsP₃-induced Ca²⁺ release.

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Penguins disturbed by tourists

SIR — Nimon *et al.*¹ found no differences in the heart rate of gentoo penguins (*Pygoscelis papua*), measured by using an artificial egg, in the absence and presence of one person. They concluded from this observation that our recorded increases in Adélie penguin (*P. adeliae*) heart rate during human presence were primarily a consequence of our² experimental method. They suggested that our birds had been stressed while we fitted them with the recording apparatus and that they were "predisposed to extreme reaction on subsequent sighting of humans".

The attempt by Nimon *et al.* to minimize stress and provide more realistic controls, made possible by recent advances in electronic technology, is laudable. Implanted birds are indeed more likely to react adversely to humans than unhandled controls. But we believe that the interpretation of their preliminary results cannot yet be definitive with regard to the effects of tourism, and that the comparison of their data with those obtained by us on a different species is not justified.

Between November and February (during the entire reproductive season of Adélie and gentoo penguins), tourism in the Antarctic consists mainly of luxury liners landing groups of up to 100, and sometimes more, people ashore near penguin breeding colonies by means of zodiacs. Some colonies are visited as often as twice a week. It is unrealistic to expect even well-informed and well-meaning tourists somehow to spread themselves out so that only one subject can be seen by any one penguin at a time. Tourists tend to take as many photographs as they can over as wide an area as possible during the limited time they have on land.

By re-analysing the penguin heart-rate

data of Nimon *et al.*, we found that maximum values observed when humans approached were on average 19% higher than resting values of undisturbed birds. This indicates that the penguins studied by Nimon *et al.* did indeed react to the minimal human stimulus presented. There is also evidence that other bird species, for example terns (*Sterna paradisaea*), react to approaching humans by increasing their heart rates, although, as in the study of Nimon *et al.*, these birds had never been handled³.

Nimon *et al.* determined heart rates of gentoo penguins during incubation. We obtained heart rates of Adélie penguins when the chicks were in crèches, and were able to show that it is not possible to extrapolate the reaction of incubating birds to other stages of breeding⁴. Escape behaviour of unhandled Adélie penguins to an approaching human was, in fact, minimal during the incubation period from November to early December and maximal when the chicks were in crèches in late January⁴. The minimum approach distance required to elicit escape from the nest increased during this period from 0.5 to 6 m. Modification of Adélie penguin behaviour and escape reactions when approached by humans on foot have also been reported by others⁵.

1. Nimon, A. J., Schroter, R. C. & Stonehouse, B. *Nature* **374**, 415 (1995).

2. Cullik, B., Adelung, D. & Woakes, A. J. in *Antarctic Ecosystems: Ecological Change and Conservation* (eds Kerry, K. R. & Hempel, G.) 177–182 (Springer, Berlin, 1990).

3. Neebe, B. & Hüppop, O. *Artenschutzreport* **4**, 8–14 (1994).

4. Wilson, R. P. *et al.* *Polar Biol.* **11**, 363–370 (1991).

5. Aguirre, C. & Acero, J. M. in *Workshop on Researcher-Seabird Interactions* (eds Fraser, W. L. & Trivelpiece, W. Z.) 41 (Fraser, Montana State Univ., 1994).

6. Bost, C. A. & Clobert, J. *Acta oecol.* **13**, 593–605 (1992).

Although Adélie and gentoo penguins are congeneric, interspecific and even intraspecific differences can be substantial. For example, gentoo penguins breeding at Ardley island (South Shetlands), very near to a summer station, can easily be approached to 1 m by a single human. In this respect, they are similar to the birds studied by Nimon *et al.* However, gentoo penguins at the small, undisturbed Hope Bay (Antarctic peninsula) colony will abandon their nests when approached slowly to 20 m by a single human (our unpublished observations). The same is true for sub-Antarctic gentoo penguins⁶, a situation which presumably renders ineffective a heart rate measuring system encased in an egg. With such substantial intraspecific differences, it is premature to make interspecific generalizations.

Finally, it is not only penguins near nests that are likely to be disturbed by tourist activities. We observed that a single human approaching to within 20 m of unhandled Adélie penguins commuting between the beach and their colonies in mid-January caused the birds to deviate from their normal tracks. The birds made a 70-m detour for several hours after the human had left the area. We estimated that this single disturbance caused the 12,000 birds commuting on these tracks during the 10 h of observation to cover an extra 840 penguin-km. Observation was by remote-controlled video placed 150 m from the study site⁴.

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Oil biodegradation around roots

SIR—Four years after the Gulf War, about 50 km² of the Kuwaiti desert still suffers from the intensive oil pollution caused by the Iraqi forces. Although there is experimental evidence for some self-cleaning of this environment through the activity of indigenous microorganisms¹, heavily contaminated areas still fail to support wild plants; however, moderately to weakly contaminated areas with less than about 10% by weight of oil sediments currently support such annuals.

Here we report that roots of such plants and of crop plants grown in polluted sand in pots under field conditions are associated with hundreds of millions of oil-utilizing microorganisms per gram of fresh roots. These microorganisms take up and metabolize various aliphatic and aromatic hydro-

carbons rather quickly, thus detoxifying and bioremediating the soil in the immediate vicinity of the roots. This may explain the plant survival mechanism in oil-contaminated soils. Further, this finding paves the way for a bioremediation approach which depends on densely cultivating oil-polluted desert areas with selected crop plants that tolerate oil and whose rhizospheres are rich in oil-degraders. Heavily contaminated areas would first have to be mixed with clean sand to dilute the oil to levels tolerable by the plants.

We initially observed that Kuwaiti desert plants, belonging predominantly to the family Compositae, although growing in black, polluted sand, always possessed white, clean roots. Even the sand adhering to the roots, the rhizosphere, was always clean whereas the sand just apart was black and polluted. We took complete desert plant samples to the laboratory, and also potted three crop plants, corn, tomato and termis, in sand we had previously polluted with 10% by wt crude oil. The pots were kept for 4 weeks in the Botanical Garden, under open conditions.

Whole roots of desert and crop plants, together with the adhering soil particles, were blended for 30 s in sterile water and the suspensions used for plate-counting and isolating oil-utilizing microorganisms using a solid inorganic medium containing 2% crude oil as a sole source of carbon². Representative oil-utilizing strains were isolated, purified and identified, and their potential to take up and oxidize individual alkanes and aromatic hydrocarbons was investigated using GLC analysis of hydrocarbons and cell total fatty acids, as we have described previously^{2,3}.

Seeds of the three crop plants showed a germination rate of 90–100% in the polluted pots, comparable to seeds in control clean pots. However, 4-week plants in the polluted pots, although quite healthy,

grew more weakly than the control plants, reaching 60–75% of their optimum height.

The rhizosphere samples of all plants were rich in oil-utilizing microorganisms. Respective bacterial numbers for corn, tomato and termis were 3.0×10^8 , 8.1×10^8 and 5.2×10^8 cells per g fresh roots, and fungal numbers were 4.4×10^5 , 1.5×10^5 and 1.4×10^5 propagules per g fresh roots. In all samples, filamentous actinomycetes, probably *Streptomyces*, were present at several thousand propagules per g root. One bacterial genus, *Arthrobacter*, was predominant (>95%) in the rhizosphere of all plants, whereas in the polluted non-rhizospheric soil several other genera, *Rhodococcus*, *Pseudomonas* and *Bacillus*, predominated, with *Arthrobacter* making up <5% of the total. The predominant fungi in the rhizosphere samples belonged to *Penicillium* and *Fusarium*, but in the polluted non-rhizospheric soil *Trichoderma* predominated. In a few cases an oil-utilizing yeast was found as a minor constituent of the rhizospheric microflora.

A total of eight strains of *Arthrobacter*, two of *Penicillium* and two of *Fusarium*, isolated from the rhizosphere of various plants, grew well, utilizing individual even- and odd-chain *n*-alkanes with C₁₀ to C₄₀ chains and three representative aromatic hydrocarbons, benzene, naphthalene and phenanthrene, as sole sources of carbon and energy. Four representative predominant *Arthrobacter* strains in the rhizospheres of various plants could quickly consume the *n*-alkanes dodecane (C₁₂), hexadecane (C₁₆) and docosane (C₂₂) and the aromatic hydrocarbons naphthalene and phenanthrene from their growing media. GLC analysis of the *n*-alkane-incubated biomass revealed that cells accumulated in their lipids fatty acids equivalent in chain length to the substrate alkanes: that is, the cells could metabolize oil constituents further after their uptake.

In conclusion, the rich oil-utilizing microflora around roots of crop plants obviously clean oil-polluted soil just adjacent to the roots. Our new work consolidates a few earlier reports^{4,5} by suggesting densely cultivating suitable plants in polluted Kuwaiti desert areas as a promising approach for their bioremediation. Heavily polluted areas would first have to be mixed with sand from adjacent clean areas to reduce the oil content to 10% by wt or less, the concentrations tolerable by the crops.

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1. Sorkhoh, N. A., Al-Hasan, R. H., Radwan, S. S. & Hoepner, Th. *Nature* **359**, 109 (1992).
2. Sorkhoh, N. A. *et al.* *Envir. Pollut.* **65**, 1–17 (1990).
3. Sorkhoh, N. A. *et al.* *J. appl. Bact.* **69**, 850–863 (1990).
4. Lee, E. & Banks, M. K. *J. envir. Sci. Hlth A28*, 2187–2198 (1993).
5. Thorhaug, A., Marcus, J. & Booker, F. *Mar. Pollut. Bull.* **16**, 355–360 (1985).



Individual *Senecio glaucus* plants growing along the polluted border of an oil lake in the Kuwaiti desert. The plant roots and adhering sand particles are white and clean. In contrast, the surface of the transitional zone between the root and shoot is black and polluted.

Silent legacy of the bomb

Michael O'Riordan

Suffering Made Real: American Science and the Survivors at Hiroshima. By M. Susan Lindee. *Chicago University Press: 1995. Pp. 287. \$29.99, £23.95.*

FIFTY years ago this August, the most terrible of all wars came to a terrible end. Two atomic bombs were dropped on Japan and destroyed two cities. Before the end of 1945, according to the local Committee for the Compilation of Materials, about half of the 300,000 people of Hiroshima had died and more than a quarter of the 250,000 in Nagasaki. Most of the deaths were caused by burns and blasts, some by radiation: because of the compound nature of the injuries, the proportions remain uncertain.

Conditions for the survivors were awful. The sheer horror of the devastation and suffering was overwhelming. Families, homes and places of work were lost. Medical and other services were disrupted. There had been, to use Robert Jay Lifton's searing phrase, immersion in death.

When members of the US Strategic Bombing Survey studied the effects of the atom bombs a few months later, they were not surprised to find that many of the survivors had feelings of hatred and anger towards Americans. As time passed, according to the local committee, their wrath was turned on their own government for having brought Japan into the war, and this attitude was, in turn, modulated into a call to ban all nuclear weapons.

The war had cost Japan more than 2 million lives, of which almost 0.4 million were civilian. There were 0.4 million US deaths of which 0.1 million were in the Pacific theatre. From the attack on Pearl Harbor to the surrender in Tokyo Bay, the war in the Pacific was marked by rapid and widespread initial conquest followed by gradual re-conquest with massive naval and air engagements, enormous amphibious operations, bloody battles for jungle islands, deadly submarine attacks and furious bombing of the Japanese home islands as a prelude to invasion. But the invasion became unnecessary.

Within days of the bombings, which took place on 6 and 9 August, teams of Japanese scientists and doctors entered Hiroshima and Nagasaki to establish the causes and consequences of the destruction. Among them was Yoshio Nishina, a former student of Niels Bohr and Japan's leading atomic physicist for whom the Klein-Nishina formula is named; he had been engaged in atomic bomb research for the armed forces. By inspecting the condition of the casualties at Hiroshima

and the exposure of photographic film, he confirmed that the bomb had indeed been atomic.

Radiation sickness set in early for those who were doomed to die from high doses — nausea, vomiting, anorexia, haemorrhaging, diarrhoea, fever and death. Some who lived a little longer also suffered characteristic hair loss. The

IMMERSION in death — Yosuke Yamahata was one of three men sent by the Japanese Army to document the effects of the "new style bomb" dropped on Nagasaki. His hundred or so images, examples of which appear here and on the following page, make up the most extensive photographic record of the immediate aftermath of the atomic bombings of Japan. They are taken from the quietly astounding *Nagasaki Journey*, the first publication of the Yamahata archive in more than 40 years. This bilingual edition is issued to mark the fiftieth anniversary of the Hiroshima and Nagasaki bombings and accompanies a travelling exhibition and film of the same name opening simultaneously in New York, San Francisco and Nagasaki. Pomegranate Artbooks, \$22.95, £14.99 (pbk).



plutonium bomb at Nagasaki was more powerful than the uranium bomb at Hiroshima and exploded at a lower altitude of 500 metres, so the gamma-ray doses at various distances from the hypocentre or ground zero were different. In round terms, however, doses to the whole body for unshielded people were about 15 Gy near 0.7 km and 5 Gy near 1 km. At 15 Gy, death occurs within a few days from damage to the nervous system; at 5 Gy, more than half the victims die within a few months from damage to the bone marrow; at intermediate doses, death follows within a few weeks from damage to the gastrointestinal tract and the lungs. (Gy is the symbol for gray,

the unit of absorbed dose, named after a British physicist, which indicates the amount of radiation energy absorbed in tissue.)

After the surrender on 15 August, several Japanese relief and survey teams operated in Hiroshima and Nagasaki. They gave first aid, made haematological and pathological examinations and tried to establish the degree of radiation exposure of the victims. Within a month, the government formed a Special Committee for the Investigation of A-bomb Damages which was composed of doctors and scientists from many disciplines. Its purpose was to arrange surveys and collate results, but it was not allowed to function freely by the Occupation forces.

Almost as quickly, the Americans organized the Joint Commission for Investigation of the Effects of the Atomic Bomb in Japan which drew on the Japanese data for its comprehensive medical report a little over a year later in September 1946. Convinced of the need and usefulness of continuing such studies, but under civilian rather than military auspices, the Americans proposed that a presidential directive be issued to the National Research Council of the National Academy of Sciences requiring it "to undertake a long range, continuing study of the biological and medical effects of the atomic bomb on man". President Truman issued the directive on

26 November 1946.

Thus it was that the Atomic Bomb Casualty Commission (ABCC) came to be. In 1975, it became the Radiation Effects Research Foundation (RERF), but the study still went on. RERF is supported equally by the Japanese government through the Ministry of Health and Welfare and the US government through the National Academy of Sciences under contract with the Department of Energy.

Regrettably, this arrangement is now under a suspended threat because US officials wish to re-structure the funding and management (see *Nature* 375, 709; 1995). Despite being far from complete, no epidemiological study of late radia-

tion: the probability of suffering the effect is about 0.4 for a dose of 1 Gy to the fetus. A progressive loss of IQ was also observed as dose increased, with a downward shift in the distribution of 30 IQ points per Gy. At high doses, the shift would shade into severity.

But the most resonant finding is the lack of hereditary disorders in children of exposed parents. A cohort of 31,000 children born to parents who were proximally exposed and received a combined average dose to the gonads of 0.4 Gy has been followed for four decades or more with a matched control group of 41,000 born to distally exposed parents. Several indicators of genetic effects have been

Susan Lindee's book about the ABCC, originating from a doctoral dissertation in 1990 at Cornell University, is a compelling — and disturbing — read. Drawing heavily on archive material at the National Academy of Sciences, she develops an assessment of the ABCC and its work that is essentially negative, and an appraisal of the US role in it that is often captious. There is throughout an undertone of distaste for the whole undertaking.

Her thesis is that the work was unduly influenced by the political and social circumstances, that the survivors were victimized by the research, that the failure to treat them was inhumane and that the Americans practised a form of colonial science. She repeats J. B. S. Haldane's cleverly cruel comment that working for the ABCC would be as difficult as taking part "in a joint Roman-Jewish study of the physiological effects of crucifixion".

A sizeable part of the book is given over to an assessment of the early genetics programme. The programme began in 1946, the year H. J. Muller received the Nobel prize for his work on mutation by radiation, so professional and public interest was intense. Lindee expresses reservations about the design and execution of the study, particularly about the endpoints selected and the methods of monitoring them. She makes much of the uncertainties in the results and of the difficulties researchers had in developing a consensus on them. When she comes to the classic 1956 monograph by James V. Neel and William J. Schull at the ABCC, which indicated that no conspicuous genetic sensitivity to radiation was emerging, she finds the outcome troubling "given the real public concern about the biological effects of radiation".

From time to time, brief descriptions of the early days of the ABCC by some of the founding scientists — US and Japanese — appear in *RERF Update*, the quarterly newsletter from Hiroshima. They do not conceal the tensions at the beginning of the project, but the newsletter reveals the closeness of the collaboration that has developed over the decades. The most illuminating and deeply felt account of the science in its cultural context is, however, Schull's book *Song Among the Ruins* (Harvard University Press, 1990). A veteran of the US forces in the Pacific, Schull has received the highest imperial award bestowed on those who are not citizens of Japan for his participation for more than four decades in the ABCC-RERF studies. His book is suffused with mature understanding. It is an affecting counterpoint to Lindee's. □

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Photograph by Yosuke Yamahata, from *Nagasaki Journey* (see preceding page).

tion effects has been so informative and influential as that of the atomic bomb survivors. Survivors are defined as people eligible for recruitment to the study because of fairly reliable estimates of dose. They were either proximally or distally exposed, the boundary being 2 km from ground zero in both cities, where the dose was about 0.1 Gy, roughly the same as the lifetime dose from natural gamma and cosmic rays. The children of survivors have also been studied.

The most important finding is of excess fatal cancers among the survivors themselves. Of the cohort of 76,000 or so with good dosimetry, about half are still alive. In the first four decades, some 400 more deaths than expected were observed from malignancies in a round total of 7,000. A new batch of data is due soon, but the available information has been used by various authorities such as the International Commission on Radiological Protection to project the risk coefficients for cancer in such circumstances: it is about 10 per cent per Gy.

Perhaps the most poignant finding is of mental retardation among those exposed in the womb. In a cohort of around 1,500, 30 cases of severe mental retardation were observed, with the highest incidence among those exposed between 8 and 15 weeks after concep-

tioned, including adverse pregnancy outcome, mortality, cancer, chromosome abnormalities and protein mutations: no statistically significant effect of parental exposure has been detected. It has been possible, however, to use the negative findings to estimate that the doubling dose for defects is about 2 Gy. This implies that an instantaneous gonad dose of 2 Gy may double the spontaneous mutation frequency in a human population. According to the United Nations Scientific Committee on the Effects of Atomic Radiation, the natural prevalence of genetic disorders, excluding multifactorial diseases, is about 6 per cent in populations generally.

All of these findings, appropriately adapted to the ordinary circumstances of human exposure, underpin the policy and practice of radiological protection around the world. The universal dose limits applied in industry, medicine, research and the environment are determined by the evolving results of the epidemiological studies of the survivors and their children. Begun in the stricken cities soon after hostilities ended, by scientists who had been caught up in the war on opposing sides, and pursued by some of them ever since, the enterprise has been invaluable and the *rapprochement* inspiring.

World webs

Andrew Goudie

Geocology: An Evolutionary Approach.

By Richard John Huggett. Routledge: 1995. Pp. 320. £50, \$65 (hbk); £16.99, \$24.95 (pbk).

PHYSICAL geography, at least in its crudest form, often used to be characterized by various tendencies that included description, classification and compartmentalization. Typically, a textbook would have a series of discrete chapters (on, for example, soils, vegetation and landforms) that had all too little relationship to one another.

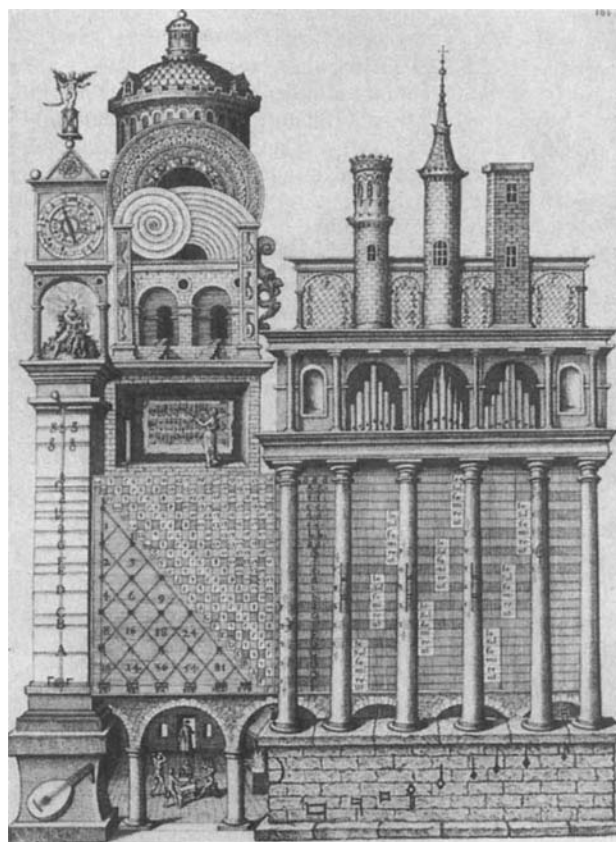
In the past two decades, physical geography has been transformed and has regained an interest in one of the fundamental tenets of the discipline: interrelationships. There are various reasons for this transformation, among them the adoption of systems-thinking, the worry about the many ramifications of global and more local environmental changes (whether naturally or anthropogenically induced) and a more balanced concern about the different components of physical geography (leading to a resurgence in biogeography).

Richard Huggett has written a series of books relating to these developments, and *Geocology* is his latest. The message of this well produced and well written book is that not only do animals, plants and soil interact with one another, but they also interact with the atmosphere, hydrosphere, troposphere and biosphere to produce landscape systems or geoeosystems. Geocology is the study of the structure and function of geoeosystems at a variety of scales. The core of the book relates to the "internal influences" that mould geoeosystems, and explores the links between climate and soils, climate and life as well as the influence of altitude, substrate, topography and insularity. It concludes with a consideration of "external influences" (that is, types of disturbance).

The book is geared towards "upper level students and academics". Those taking and teaching courses in physical geography or environmental science will greatly profit from using it, as long as they have first assembled some of the building blocks of their trade from the older genre of textbooks. One of the dilemmas for university teachers of physical geography is that some of the school syllabuses increasingly produce students who have not assembled the building blocks to allow them to gain the stimulus that they could and should get from this informative, intriguing and idiosyncratic volume. □

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The harmonies of nature



ROBERT FLUDD, the seventeenth-century English doctor and occult philosopher, wrote a series of vast and beautifully illustrated treatises in which he attempted to reveal the underlying harmony and congruity between the Universe (the "macrocosm") and man (the "microcosm"). This picture, "The Temple of Nature", was intended to be a mnemonic aid that enabled adepts to memorize the basics of music theory. It is taken from *The Music of the Spheres* by Jamie James, an elegantly written reassessment of the Western musical tradition and its relation to science. First published in 1993, the book is now issued in paperback by Copernicus, a new imprint of Springer (New York). Price is \$13.

The uses of mathematics

C. W. Kilmister

On the Shoulders of Giants. By Malcolm E. Lines. Institute of Physics Publishing: 1994. Pp. 288. £49.50, \$90 (hbk); £15, \$30 (pbk).

THIS book sets new standards in the popularization of both mathematics and physics. The author has been motivated to write by his puzzlement over the "unreasonable usefulness" of mathematics in physics, and it is the driving force of this puzzle that makes the book so good. Although the author has no answers to the problem, he successfully displays, for those with only a modest knowledge of either subject, 12 historical perspectives that show its acuteness.

All the stories he tells make the point well, but naturally some are less exciting than others. "From Euclid to general relativity" is a fairly standard run through the discovery of non-Euclidean geometries, and "From integers to quaternions" has little physical content beyond the representation of spatial rotations, which had

been part of the mathematical drive anyway. But the best of the others are gems; I particularly like "From Aristotle to the structure of glass", which begins with a careful explanation of the problem of packing space with regular solids and the long history of trying to do so with regular tetrahedra, and which then, after a good description of crystallography, goes on to the problem of closest packing of spheres and so on to glasses. This chapter is nicely complemented by one on tiling, which takes the reader through irregular tiling to quasicrystals. The author does not neglect the latest fashions of chaos, fractals and superstrings and he has something new to say about probability and topology.

The treatment of topology in fact illustrates just how well the book succeeds, with the author tracing his way from the familiar bridges of Königsberg through the four-colour theorem to space-filling polyhedra (Voronoi polyhedra) and finally to polymers and glasses again. Such cross-referencing gives the book a satisfying unity. □

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Three lemming puzzles

Ilkka Hanski

The Biology of Lemmings. Edited by Nils Christian Stenseth and Rolf Anker Ims. Academic Press: 1994. Pp. 683. £40, \$99.95.

WHEN the 23-year-old Charles Elton passed through the Norwegian town of Tromsø on his way back from an Arctic expedition in 1923, he spent much of his remaining money on a book by Robert Collett on Norwegian mammals. Elton

(D. Wilson and D. Reeder, *Mammal Species of the World*, 2nd edn, Smithsonian Institution Press, 1993). To their credit, however, they present an informative discussion on the genetic diversity in the problematic genus *Dicrostonyx*.

Of the three lemming puzzles, that of population dynamics receives most attention. So much has happened recently in the study of lemming dynamics and in rodent dynamics generally that the general framework provided here has become out of date. The current excitement about nonlinear rodent dynamics and the new approaches to modelling and data analysis are barely touched on in these pages.

Definite high points in this volume

IMAGE
UNIMAGEABLE
FOR A COPYABLE
REASON
REASONS

The Norwegian lemming, which, contrary to popular belief, is the only lemming species that migrates.

understood enough of the Norwegian text to be struck by the regularity in the year-to-year fluctuations of the numbers of lemmings. Since then, ecologists have been puzzled by the 'lemming cycle' and, following the lead of many illustrious naturalists and laymen, by the proverbial lemming migrations. The third lemming puzzle is extraordinary sex ratios in some lemming species.

The three puzzles have now been brought together in a book edited by Nils Stenseth and Rolf Ims. With contributions from 30 or so authors, it is undoubtedly the most thorough treatment of lemmings since Elton's *Voles, Mice and Lemmings* (1942), lacking only a thorough coverage of the Russian literature. Like many other symposium volumes, *The Biology of Lemmings* will appeal to the expert for its rich mixture of miscellaneous information, but it may occasionally frustrate the more general reader for the same reason. Long introductions to each of the six sections go some way in presenting a summary and synthesis, although they also give the impression of having exhausted the editors. It is unfortunate that the volume took seven years to mature. In the meantime, things have changed, including the number of lemming species recognized. G. Jarrell and K. Fredga arrive at ten species, but many more are now listed

include an historical overview of the beliefs, knowledge and research on lemmings (Stenseth and Ims), a thorough description of lemming movements in northern Fennoscandia (H. Henttonen and A. Kaikusalo) and new results on the ecology and genetics of skewed sex ratios in the wood lemming (Fredga and co-workers). Of the three lemming puzzles, that of sex ratios seems nearest to being solved. Earlier ideas about inbreeding in spatially structured populations are not supported; instead the evidence points to large fitness differences among the three types of females.

The myth of lemming migrations seems to be a myth for every species except the colourful Norwegian lemming in parts of Fennoscandia. (Why the Norwegian lemming is so colourful is a small wonder in itself.) Here the Arctic migrations present a unique spectacle, though only at roughly 30-year intervals. During a mass migration, millions of lemmings move at a speed of 5 km per hour, covering distances up to 200 km. For those who don't believe without seeing, let it be known that the next mass migration is due around the turn of the century. □

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New in paperback

The Dictionary of Cell Biology edited by J. M. Lackie and J. A. T. Dow (2nd edn). Academic, £15. First published in 1990, this revised edition has more than 1,000 new entries, bringing the total to over 5,000. For a review see *Nature* **343**, 225 (1990).

Beast and Man: The Roots of Human Nature by Mary Midgley (revised edn). One of the first critical examinations of sociobiology, written from the perspective of a moral philosopher. Routledge, £12.99.

The Theory of Gambling and Statistical Logic by Richard A. Epstein (revised edn). First published in 1977, this classic work covers the full range of games. Witty and informative reading for anyone planning to teach a course in probability or manage a casino properly. Academic, £23.

Life in the Universe: Scientific American — A Special Issue. The 10 chapters and the epilogue of this book originally appeared as articles and the closing essay in the October 1994 issue of *Scientific American*. Contributors include Steven Weinberg, Leslie E. Orgel, Stephen Jay Gould, Carl Sagan, William H. Calvin, Marvin Minsky and Antonio R. Damasio. W. H. Freeman, £14.95.

Anthropology and Politics: Vision, Traditions, and Trends by Joan Vincent. A scholarly look at the changing social and political contexts in which the subject has operated, and on how these external forces have shaped its development. University of Arizona Press, \$21.50.

The Transition from Infancy to Language: Acquiring the Power of Expression by Lois Bloom. The author summarizes the results of a 10-year study of 14 children. Cambridge University Press, £13.95, \$16.95.

In Search of the Edge of Time: Black Holes, White Holes, Wormholes by John Gribbin. More thought-provocation from one of Britain's most prolific science popularizers. Penguin, £6.99.

Seven Ideas that Shook the Universe by Nathan Spielberg and Bryon D. Anderson (2nd edn). A popular romp through Copernican astronomy, Newtonian mechanics, energy, entropy and probability, relativity, quantum theory, and conservation principles and symmetry. Wiley, £17.95.

Photodissociation Dynamics by Reinhard Schinke. "Schinke has amply fulfilled his declared aim of providing an overview of the field for graduate students in molecular physics and for experimentalists who are not familiar with the quantum theory of photochemistry", said Richard N. Dixon in *Nature* **366**, 120 (1993).

Bruce Coleman / Alan G. Potts

Protein molecules as computational elements in living cells

Dennis Bray

Many proteins in living cells appear to have as their primary function the transfer and processing of information, rather than the chemical transformation of metabolic intermediates or the building of cellular structures. Such proteins are functionally linked through allosteric or other mechanisms into biochemical 'circuits' that perform a variety of simple computational tasks including amplification, integration and information storage.

In unicellular organisms, protein-based circuits act in place of a nervous system to control behaviour; in the larger and more complicated cells of plants and animals, many thousands of proteins functionally connected to each other carry information from the plasma membrane to the genome. The imprint of the environment on the concentration and activity of many thousands of proteins in a cell is in effect a memory trace, like a 'random access memory' containing ever-changing information about the cell's surroundings. Because of their high degree of interconnection, systems of interacting proteins act as neural networks trained by evolution to respond appropriately to patterns of extracellular stimuli. The 'wiring' of these networks depends on diffusion-limited encounters between molecules, and for this and other reasons they have unique features not found in conventional computer-based neural networks.

Components

In principle, any protein that transforms an input signal into an output signal could act as a computational or information-carrying element. Thus an enzyme in a biochemical pathway 'reads' the concentration of its substrate and produces a corresponding level of product; a receptor on a cell surface reads the concentration of its ligand and produces a certain level of receptor-ligand complex (Fig. 1a). Simple enzymes and receptors generate a monotonic relationship between input and output, which saturates as the concentration of substrate or ligand rises. A more abrupt and switch-like performance is shown by many proteins, typically those composed of multiple subunits, where interactions between subunits cause the rate of enzyme reaction or ligand binding to rise steeply in sigmoidal fashion over a limited range of concentration (Fig. 1b).

These simple (although nonlinear) relationships are made more complex by allosteric mechanisms that allow proteins to be controlled by changes not only in their levels of substrate or ligand but also in the concentrations of regulator molecules. The enzyme aspartate transcarbamoylase, for example, is composed of multiple catalytic and regulatory subunits, and flips in an abrupt, cooperative fashion between an active and an inactive state. Binding of its substrates (aspartate and carbamoyl phosphate) drives the enzyme into an active conformation in which it makes *N*-carbamoylaspartate and hence begins the synthesis of the pyrimidine ring of C, U and T nucleotides. By contrast, binding of cytidine triphosphate (CTP), one of the end products of the pathway, converts the enzyme into an inactive conformation from which the substrates dissociate. The rate of reaction of this enzyme is consequently highly sensitive to variations in concentration of three input molecules¹ (Fig. 1c).

The activity of a protein can be altered not only by binding to a regulatory molecule but also by enzyme-catalysed modification. Many proteins are chemically modified after synthesis by the covalent addition of chemical groups such as methyl, nucleotidyl, fatty acyl, myristoyl and, particularly, phosphoryl groups, changes that, in general, alter the biological activity of

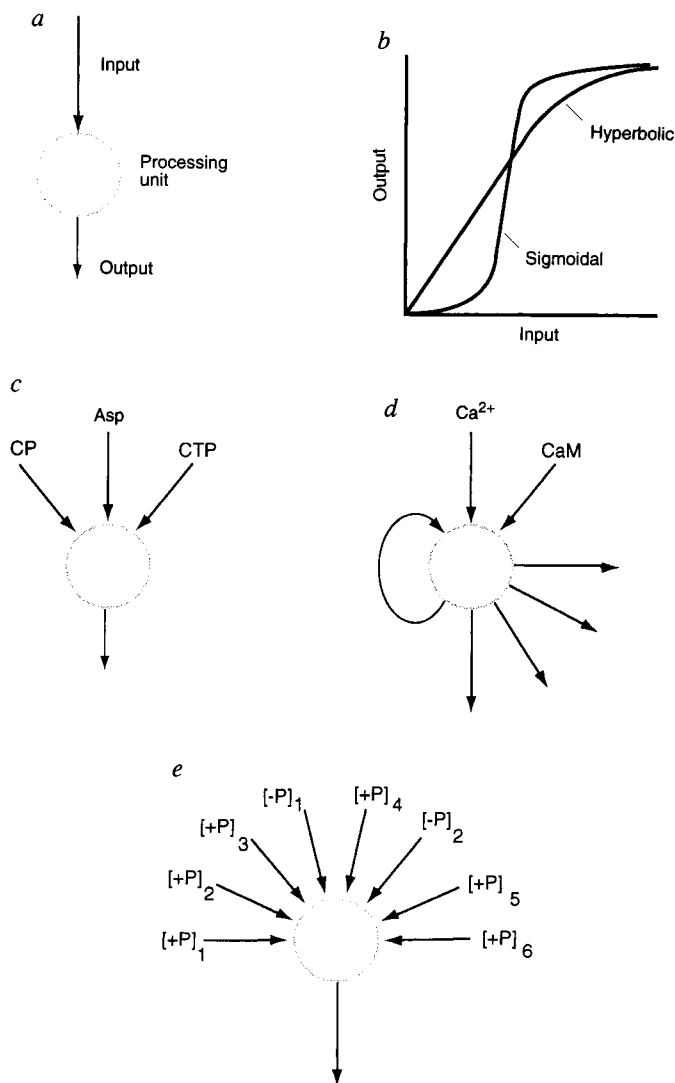
the protein. In some cases protein modification changes the three-dimensional structure of the protein and, by altering the interactions with adjoining subunits, thereby causes a cooperative, or sigmoidal, response. The enzyme glycogen phosphorylase, for example, could be represented symbolically in a similar way to aspartate transcarbamoylase in Fig. 1c simply by replacing the inputs with (1) the concentration of its substrate, glycogen, (2) the activity of phosphorylase kinase, which adds a phosphate group to a site that is remote from the catalytic site, and (3) the activity of a protein phosphatase that removes this phosphate group².

Post-translational modification allows a single species (the modifying enzyme) to influence the activity of many others. Thus a change in activity of a protein kinase typically affects multiple target proteins. The action of a protein kinase may also be made more complex if it works back on itself to phosphorylate its own amino acids. Proteins that show this intramolecular feedback sometimes appears to act as an irreversible switch, as seen in the calcium-sensitive enzyme Cam II kinase (Fig. 1d). This protein, abundant in brain and concentrated in synaptic terminals, is stimulated by an increase in the level of cytosolic Ca^{2+} ions in the presence of calmodulin, leading to the phosphorylation of multiple target proteins. Among these target proteins is the Cam II kinase itself which, when it attains a sufficiently high level of phosphorylation, becomes irreversibly active regardless of the level of Ca^{2+} . This enzyme is indeed a sophisticated molecule, for its level of activity depends not only the size and number of Ca^{2+} spikes but also their frequency³. It is believed that this protein may be important for neuronal processes, such as synaptic plasticity, that depend on frequency-dependent modulation by Ca^{2+} spikes⁴.

Analogous processing is seen in the membrane receptors for growth factors such as platelet-derived growth factor (PDGF) and insulin. When these receptors bind their ligand they associate into dimers, which activates a kinase associated with their cytoplasmic domains. Each kinase then phosphorylates, in reciprocal fashion, its sister domain on crucial tyrosine residues. These phosphorylated tyrosines are subsequently recognized by other proteins in the cytoplasm which bind to the sequence containing the modified tyrosine and hence change their own activity. In this way, information that the cell has encountered a specific hormone or growth factor is distributed along multiple divergent paths^{5,6}.

Phosphorylation also provides the basis of integration whereby a single protein combines multiple inputs to produce a single output in a manner analogous to the integration by a nerve cell of multiple synaptic inputs. This type of integration is seen in the product of the *c-src* gene, an intracellular signalling protein that is itself a kinase⁷, and in glycogen synthase, the enzyme that synthesizes glycogen in a liver or muscle cell⁸. The latter protein has at least ten distinct sites at which phosphates can be added. Six or more different protein kinases are responsible for the addition of these phosphate groups, and one or more phosphat-

FIG. 1 Proteins as computational elements. **a**, A simple protein shown as a processing unit with the flow of information indicated by an arrow. The symbol used here is based on a perceptron, which is a device used to portray the processing of synaptic inputs by a nerve cell. For a simple enzyme, the input would be the concentration of its substrate and its output catalytic activity. For a membrane receptor, the input would be the concentration of ligand and the output the level of receptor occupancy. **b**, The input/output relationship of a simple enzyme or receptor is typically either hyperbolic or sigmoidal. **c**, Aspartate transcarbamoylase, a classic example of an allosteric enzyme. The activity of the enzyme is controlled by the concentration of its two substrates, carbamoyl phosphate (CP) and aspartate (Asp) in combination with the concentration of an end product, cytidine triphosphate (CTP), of the biosynthetic pathway initiated by the enzyme. **d**, Cam II kinase, a molecular switch, becomes active when both calcium ions (Ca^{2+}) and calmodulin (CaM) are present. The enzyme phosphorylates multiple target proteins including itself. When it reaches a sufficiently high level of phosphorylation, the enzyme switches to a permanently active state that is independent of the concentration of Ca^{2+} . **e**, Glycogen synthase, the enzyme that makes glycogen, is phosphorylated on multiple sites through the action of six protein kinases (+P) and several protein phosphatases (-P).



ases remove them. The target protein in this example serves to integrate the action of multiple modifying enzymes (Fig. 1e).

Living cells contain an enormous diversity of protein structures and the signals they carry are not limited to the binding of molecules or covalent modifications. Thus many proteins take as their 'input' contact with a macromolecule such as another protein or a molecule of DNA or RNA. Proteins are also known that respond specifically to light, to temperature, mechanical forces or voltage. The 'output' of a protein is just as diverse, and may be the formation of a macromolecular structure, the generation of a physical movement or production of light. Each input/output relationship is kinetically distinct, usually non-linear, and often extremely rapid. Dynamic aspects of protein processing have been analysed in greatest detail in the behaviour of ion channels in the plasma membrane. Physiological recordings using patch-clamp electrodes show that a typical voltage-gated or ligand-gated ion channel—structures built from several protein molecules embedded in the plasma membrane—has multiple distinct states and changes from one to the next in less than 50 μsec (ref. 9). In principle, such a channel could carry information at a substantial rate.

Building circuits with proteins

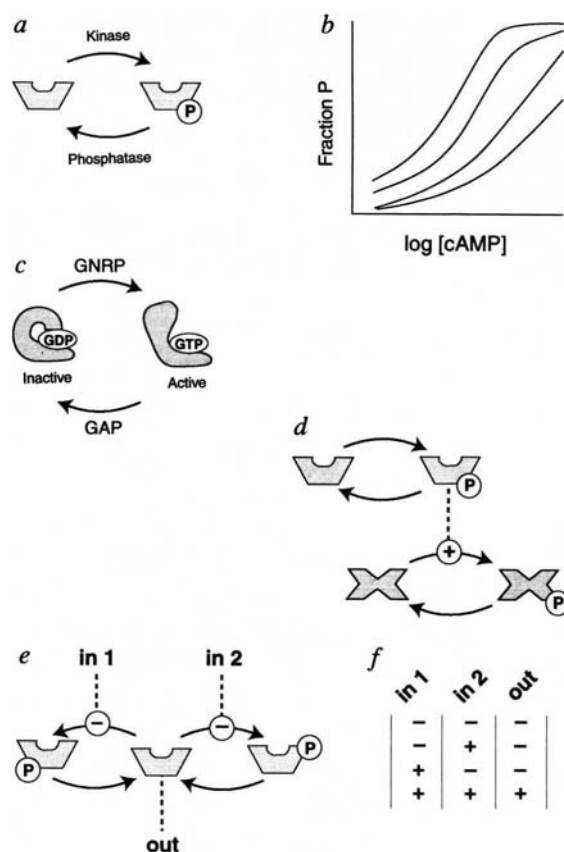
In an important theoretical paper¹⁰ in 1943 on 'artificial intelligence', the neurophysiologists McCulloch and Pitts demonstrated that small numbers of idealized nerve cells, linked into circuits,

can perform a variety of logical operations with no obvious upper limit to their complexity. Although it was recognized from the start that these devices ('McCulloch-Pitts neurons') were artificial and highly simplified, there was no reason, it was argued, why real nerve cells in the brain should not be capable of performing equally complex logical operations. Few today would disagree with this thesis.

As with neurons, so with proteins. Putting aside for the moment the question of whether it is useful or even sensible to view them in this manner, it is nevertheless true that protein molecules are in principle able to perform a variety of logical or computational operations. The symbols used in Fig. 1 to depict the operation of individual protein molecules are based on another, slightly more flexible, type of neuron-like unit, called a perceptron^{11,12}. Thus protein molecules provide a cell with a toolkit of components from which virtually any circuit could be built. To take a simple example: a protein that is modified at two allosteric sites by two separate kinases (or by binding two independent allosteric regulators) could perform a variety of logical operations on these two 'inputs'. If this protein is active only when both sites are phosphorylated, it will act somewhat like a boolean AND gate: if it is active when either or both sites are active, it will be like an inclusive OR gate, and so on.

The crispness of the input/output response—how closely it resembles a digital switch—will depend on the kinetic parameters of the protein, such as its cooperativity¹³. Although each indi-

FIG. 2 Cyclic reactions as computational elements. **a**, Schematic representation of the cyclic phosphorylation and dephosphorylation of an enzyme, such as pyruvate dehydrogenase or glycogen phosphorylase. Phosphorylation is catalysed by a protein kinase and driven by the essentially irreversible hydrolysis of ATP; dephosphorylation is catalysed by a protein phosphatase driven by the release of inorganic phosphate (P_i). **b**, Experimentally measured performance of a cyclic reaction. The fractional phosphorylation of a target polypeptide in a system containing cAMP-dependent protein kinase and phosphoprotein phosphatase was measured over a range of concentrations of cAMP which stimulates the kinase. Each curve shows the effect of a range of cAMP concentrations (the 'input') on the fraction of target molecules that are phosphorylated (the 'output'). Measurements were repeated at different concentrations of P_i, which inhibits the phosphatase. Data redrawn from ref. 14. **c**, The cyclic interconversion of GTP-binding proteins forms the basis of many signal-transducing steps in living cells. The protein goes through a series of allosteric transitions driven by the irreversible hydrolysis of its bound GTP. The active form of the protein, associated with GTP, triggers one of a variety of different cell processes. Progression through the cycle, and hence the rate of formation of the active species, is controlled by regulatory proteins that either catalyse GTP hydrolysis (GTPase-activating protein (GAP)) or induce the protein to release GDP, thereby preparing it for association with GTP and another round of activation (guanine nucleotide releasing factor (GNRP)). These regulatory proteins thus perform a similar function to the kinase and phosphatase enzymes in **a**. **d**, Two enzyme cycles coupled together. The phosphorylated enzyme produced in the first cycle acts as an enzyme to catalyse phosphorylation of the second enzyme. The potential for amplification achieved by the two cycles is a multiplicative function of the two individual cycles²². **e**, A reaction mechanism expected to function as an AND gate. Each of the two inputs ('in 1' and 'in 2') is assumed to inhibit one of the two protein kinases; when both are present, the concentration of A ('out') reaches its highest possible level. **f**, Given a suitable selection of rate constants and concentrations, a sharp ON response will be achieved only when both inputs are present. The addition of more enzyme cycles coupled to this one can create other logical devices, such as NOT and XOR¹³.



vidual protein molecule can exist in (usually) only two distinct conformations, the performance of a large assembly of such molecules depends on the probability that each molecule will occupy one or other of these conformations. For this reason, a different type of computational element in which the interconversion of two forms of a protein is energy driven is often able to give sharper and more easily controlled responses (Fig. 2a). Thousands of cyclic interconversions coupled to the hydrolysis of a molecule such as ATP occur in eukaryotic cells, each with the potential of acting as a molecular switch. In 1977, Stadtman and colleagues^{14,15} saw that the cyclic interconversion of a protein between its phosphorylated and non-phosphorylated forms offers a flexible computational unit capable of very rapid responses. They demonstrated, first by theoretical analysis and later by actual experiment, that the input/output response of such cycles can be modulated over a wide range by changes in rate constants and metabolite levels (Fig. 2b)^{14,15}. Sharp, digital-like responses can be attained if the target enzyme is highly cooperative or if the modifying enzymes are working near to saturation with respect to their substrates¹⁶.

Some of the more subtle features of such switches have been recently explored by Arkin and Ross¹³, who analysed the conversion of fructose-6-phosphate between two biphosphate forms by specific kinases and phosphatases. Control of the enzymes in this kinetic mechanism, through multiple allosteric regulators, serves to switch the pathway from glycolysis to gluconeogenesis according to the energy status of the cell. The operation of this switch, simulated either in isolation or in the context of a large model of glycolysis and the citric acid cycle, was substantially richer than that of a simple ON/OFF switch. Asymmetries and synergisms between the various inputs caused the gate to operate in some situations as a reasonably smooth AND gate, and in others as an OR gate, thereby conforming to a more general class of logic called 'fuzzy logic'¹³.

Many proteins cycle between structural states that differ not in phosphorylation but in their association with a nucleoside triphosphate. Directed movements in living cells, for example, are driven by the cyclic hydrolysis of ATP catalysed by motor proteins such as myosin or kinesin. Many intracellular signals are carried by the cyclic, enzyme-catalysed hydrolysis of GTP (Fig. 2c). GTP-hydrolysing proteins are abundant in eukaryotic cells and used in many different processes, such as directing vesicular traffic through different membrane compartments of the cell and controlling the accuracy of protein synthesis^{17,18}. The small GTP-binding protein, Ras, is an enabling switch for a cascade of protein kinases, and has a wide influence on many aspects of cell growth and differentiation^{19,20}. The same fundamental cycle, in more complex form and involving more molecules, occurs in the heterotrimeric G proteins, which transduce sensory and hormonal stimuli across the plasma membrane in almost all eukaryotic cells²¹.

It is possible to link two or more cycles together by making the output of one the input, or allosteric regulator, of the next. There is a question of matching here: the frequency and amplitude of an input cycle must be within the useful range of the next cycle in the series. If it is then systems of considerable complexity may be built. Sequential cascades can generate large amplifications, for example²² (Fig. 2d), or create circuits that perform specific logical functions. The reaction mechanism shown in Fig. 2e would function as a boolean AND gate (Fig. 2f), and others can be devised that perform like OR, XOR and NOT gates¹³. Evidently this process may be continued indefinitely, so cyclic reactions could be put together to perform highly complex logical or computational functions. Indeed, it has been shown by formal analysis that cyclic chemical reactions can be used to construct a bistable system with similar properties to a McCulloch-Pitts neuron²³. Sets of chemical neurons may then be linked through coupling reactions to build logic gates and a

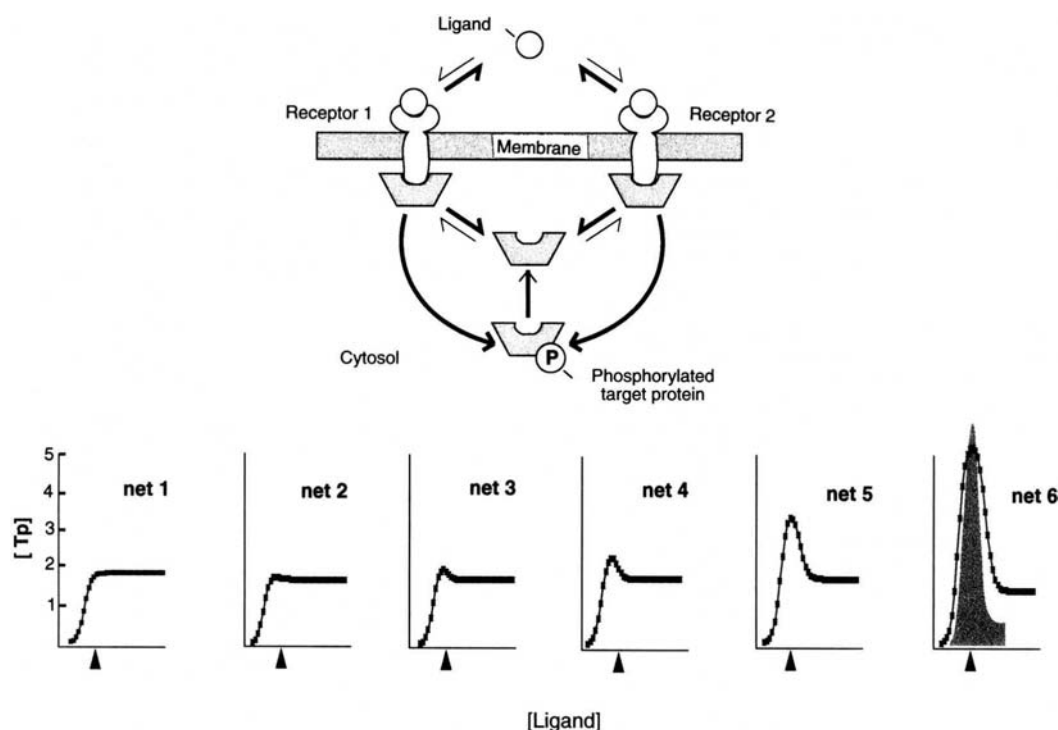


FIG. 3 Training a protein circuit by simulated evolution²⁵. **a**, Model circuit built from two membrane receptors and an intracellular target protein. The two receptors, initially identical, have a binding site for the same extracellular ligand. When complexed with the ligand, the receptors bind to the target protein in the cytoplasm and phosphorylate it. The performance of the circuit, modelled by computer simulation, is specified by the seven rate constants shown as bold arrows. **b**, Optimization of the protein circuit to produce a specified input/output relationship. The circuit shown in **a** was assigned starting values of rate constants and the amount of phosphorylated target protein (output) measured for a range of ligand concentrations. This gave the dose-response curve net 1. Random changes in the rate constants were then made after which the response of the new circuit was again tested over a range of ligand concentrations. If the new circuit gave a dose-

response curve closer to the desired one (in this case a response curve with a maximum at a specified concentration of ligand indicated by arrowheads) then this circuit was adopted in place of the preceding one. Outputs of a succession of new circuits, each closer to the target performances, are shown in net 2 to net 6. The target dose-response curve for the series is indicated by the shaded curve in net 6. This above simulation, run for a variety of different starting parameters, always achieved the same kind of solution, in which the two receptors had different affinities. The high-affinity receptor phosphorylates the target protein at a high rate, whereas the low-affinity receptor binds to the target protein but does not phosphorylate it. Some specific pairs of high- and low-affinity receptors in actual cells may have arisen by a similar process during evolution.

finite-state machine that generalizes to a universal Turing machine²⁴.

As the number of proteins in a multicyclic system grows larger, so the task of juggling its many independent rate constants and concentrations to achieve a desired input/output performance becomes increasingly difficult. Even a single cyclic interconversion may have ten or more independent variables¹⁵. A practical approach that involves biology is to use a form of optimization in which rate constants and binding constants are changed randomly until the system as a whole performs in a selectively advantageous way²⁵ (Fig. 3). It is not hard to imagine that the functional networks in living cells could have been shaped by a similar process taking place during evolution.

Protein circuits in living cells

Protein molecules can in some sense act as logical elements, and small sets of proteins can be artificially linked to perform simple computations, but whether it is useful from a biological standpoint to think of them in these terms is more debatable. Cells do not perform calculations for their own sake but as a means of monitoring and responding to their internal and external environments. Many proteins in a living cell are used to build

macromolecular structures, produce movement, degrade unwanted molecules, or synthesize specific chemical species. To liken such proteins to computational devices is inappropriate, notwithstanding the fact that they are often allosteric and subject to regulation by the mechanisms discussed above. However, there also exist biochemical pathways, such as the bacterial chemotaxis pathway described below, the primary function of which is to transfer and process information. To describe the proteins in these pathways as 'chemical catalysts' or 'protein machines' is also inappropriate. The most useful analogy in this case is that of neuron-like elements that use a weighted sum of their inputs to produce one or more outputs. Of course, this analogy, like the others, is artificial and does not concern the cell. In a living cell the molecular interactions that regulate chemical catalysis and directed motion merge seamlessly with those involved in the transmission of information.

What kinds of protein-based circuit can be considered 'computational' or 'information processing'? An attempt to answer this question uncovers a plethora of physiological responses and biochemical pathways that for the most part are incompletely understood at the molecular level. Responses of living cells to their environment demonstrate their sophisticated processing of

environmental signals, and the multitude of intracellular signalling pathways offers abundant opportunities to construct computational circuits. A typical vertebrate cell has (at a guess) several hundred different channels and receptors on its surface, dozens of different G proteins and second messengers, and hundreds of different protein kinases. These elements control the concentration or activity of many thousands of target proteins in the cell (recent estimates suggest that as much as one-third of the proteins in a eukaryotic cell are phosphorylated, for example). In only a few instances are the molecular interactions of local circuits known in sufficient detail to make a quantitative prediction of performance.

Two of the most common responses of living cells to external stimuli are amplification and adaptation. Amplification enables a cell to transform faint and ephemeral stimuli into substantive biochemical changes; adaptation enables it to measure relative rather than absolute changes and hence respond over a wide range of input stimuli. The cyclic interconversion of proteins already mentioned can generate large amplifications both in the absolute number of output molecules produced for each input molecule (or input stimulus), and also in the rate of change of the output compared to the input²⁶. Absolute amplification occurs, for example, when a single molecule of a hormone binds to a membrane receptor and, by triggering a protein kinase or activating a G protein, produces a change in many target molecules. Huge amplifications can in principle be achieved by cascades of cyclic interconversions coupled together: bleaching of a single molecule of rhodopsin in the membrane of a vertebrate photoreceptor, for example, can cause hydrolysis of 10^5 molecules of cyclic guanosine monophosphate (cGMP)^{27,28}. In general, however, only a limited degree of amplification is desirable, and chains of signals commonly use both negative amplification (diminution) as well as positive amplification²⁶. The other kind of amplification (in which small percentage changes in input produce larger percentage changes in output) can be obtained, as already mentioned, by the cooperativity of individual proteins, by coupling cyclic systems together or by enzymes that work close to saturation with respect to their substrates.

Adaptation lags behind the stimulus, following a time-course that approximates to the first differential of the input stimulus. Various mechanisms are used by cells to produce adaptation, including the reduction in number of membrane receptors, their desensitization, or the inhibition of one of the enzymes in the amplification cascade. A system in which both amplification and adaptation are sufficiently well understood at the molecular level to analyse it in terms of molecular circuitry²⁹ is that of the response of coliform bacteria to chemical attractants and repellents³⁰. Here the amplification is due to an intracellular cycle of protein phosphorylation combined with a highly cooperative interaction with the flagellar motor. Transmission of the signal from the receptor to the flagellar motor is extremely rapid—less than 0.1 s—and is probably limited by diffusion. Adaptation, by contrast, is a slower process that requires several seconds to complete, and is controlled by the catalytic rate of two enzymes, one that methylates the membrane receptors and the other that removes these methyl groups. Methylation of the membrane receptors alters the strength of the signal they pass to the phosphorylation cycle (Fig. 4). This relatively simple circuit controls the swimming of the bacterium, enabling it to respond in an informed way to its chemical environment. It exemplifies how, in unicellular organisms, proteins act in place of nerve cells to control behaviour.

Bacterial chemotaxis illustrates other common features of protein circuits in living cells, such as their ability to integrate multiple inputs. Thus, in a complex chemical environment, bacteria add the influences of different attractants and repellents to determine their swimming behaviour³¹. Similarly, in the complex milieu of a developing embryo, the tip of a growth cone uses multiple cues in the extracellular matrix to select the path along which it grows³². The action of cytokines

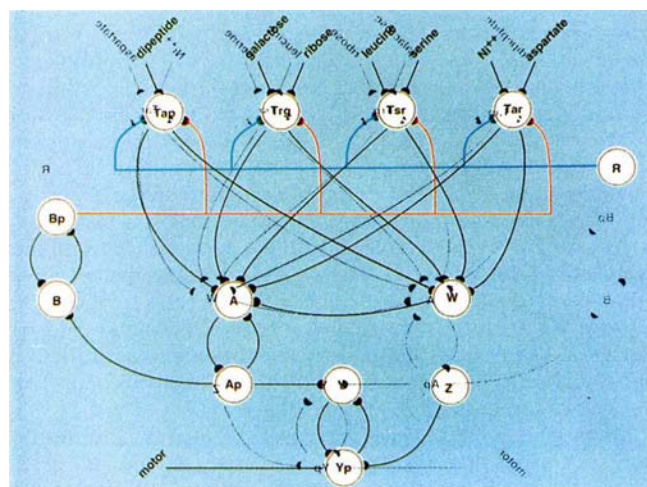


FIG. 4 Protein 'circuit' mediating the chemotactic response of coliform bacteria³⁰. Tap, Trg, Tsr and Tar are membrane receptors, each of which binds to specific attractant and repellent molecules. Binding causes a signal to pass across the plasma membrane and to change the level of phosphorylation of an intracellular protein kinase, CheA (A). The transduction mechanism involves CheW (W) which, together with CheA, forms a complex associated with the cytoplasmic domain of the receptor. Phosphate groups are passed from CheA to CheY (Y), which is also believed to be part of the receptor complex, and the phosphorylated species CheYp (Yp) then diffuses to the flagellar motor. There it changes the direction of rotation and hence modulates the swimming of the cell so that it moves towards the attractant or away from the repellent. CheZ (Z) is a phosphatase that removes the phosphate group from CheYp, thereby allowing rapid changes in phosphorylation level. Two additional Che proteins add a capacity for adaptation that is essential for the chemotactic response. CheR (R) and the phosphorylated form of CheB (Bp) are enzymes that methylate (blue lines) and demethylate (red lines), respectively, the membrane-bound receptors. Methylation counteracts the effect of the attractant or repellent by changing the strength of the signal relayed by the receptor and works more slowly than the phosphorylation cascade. The level of methylation is continually adjusted according to the recent experience of the bacterium through phosphorylation of the methyltransferase CheB by CheAp. The number of methyl groups per receptor (up to 4) changes slowly with the level of stimulation of the bacterium, producing a response that is proportional to the rate of change of the stimulus. This small network of proteins serves to measure the concentration of specific chemicals in the cell's environment, to integrate, amplify and determine the rate of change of these concentrations, and then to transmit the results of these computations to the flagellar motor. The system thus works in an analogous fashion to a group of nerve cells controlling a behavioural response in a multicellular organism. Note that at any instant of time the concentrations of the various phosphorylated proteins encode a representation, or 'memory', of the chemical environment in the past few seconds encountered by the bacterium.

on target haematopoietic cells is also characterized by pleiotropy and redundancy³³. Integration in these and other systems rests ultimately on the convergence of signals into a single signalling pathway and the generation of an activity or concentration that is a graded function of the combination of inputs. Perhaps the most astonishing evidence of this combinatorial capacity is found on the boundary of our subject (as it involves DNA molecules) in the regulation of DNA transcription in eukaryotic cells. A typical gene in a multicellular organism requires the assembly of a transcriptional complex composed of enzymes, transcription factors and gene regulatory proteins³⁴. Because these components are drawn from a very large pool of candidates, an extremely large number of different transcriptional complexes, each with a different 'blend' of proteins, is possible.

What other computational processes do protein circuits perform? A capacity for timing is evident at many levels: in the kinetic proof-reading that enhances the fidelity of protein trans-

lation (controlled by the slow hydrolysis of GTP by a protein); the detection of coincident signals³⁵; and the orderly progression of a cell through its cycle of division, governed by cascades of protein phosphorylation³⁶. Networks of proteins can also store information, not only as part of the dedicated apparatus that reinforces the effect of individual synapses, but also in a more general sense. The imprint of the environment on the concentration and activity of many thousands of molecules of the cell is in effect a memory trace. In contrast to the permanent information encoded in DNA, however, it is a 'random access memory' containing ever-changing information about the cell's surroundings. Developing organisms are shaped by protein circuits that carry signals not only from the plasma membrane to the nucleus of individual cells, but also act as diffusible inductive signals from one cell to the next.

Many aspects of cell behaviour display a capacity for information processing that is independent of the genome and hence, presumably, is controlled by sets of proteins³⁷. The oriented growth of cilia on the surface of a *Paramecium*; crawling of cells over surfaces; replication of centrioles and subdivision of the cortex in a *Drosophila* blastoderm; these are all processes that take place independently of short-term control by DNA. The electrical response of excitable cells offers another entire repertoire of input/output responses, such as integration, amplification and the encoding of inputs into a series of signals of defined frequency⁹. Although these responses are usually measured by means of ionic currents (and represented symbolically as electrical circuits), we know that they are produced by sets of allosteric proteins embedded in the plasma membrane that respond to chemical and electrical stimuli. Following this line of thought, it must be admitted that the entire short-term behaviour of any organism depends on circuits of proteins which receive signals, transduce them and relay them to neighbouring cells, occasionally producing a permanent change in the synaptic connections.

Protein-based neural networks

This review of the many parallels between protein molecules and nerve cells is not yet complete. The representation of nerve cells as symbolic devices such as perceptrons presaged the development of neural networks: computer-based models used to simulate pattern recognition and other cognitive tasks¹². Because proteins also integrate inputs and produce outputs it seems inescapable that the highly interconnected network of protein-based pathways in living cells will share some of the properties of neural nets^{24,38}. Thus we expect this cell network to recognize combinations of environmental influences and to encode abstract features of its environment in the activity of particular proteins acting as 'hidden' units. Responses of this protein-based neural network may be expected to be robust and resistant to

damage. The training of the network, which in the case of a computer-based network is achieved through the presentation of a training set of examples, would, in the case of living cells, be achieved by a darwinian process of random change and selection. Addition of new elements by gene duplication followed by independent variation would provide increasingly large networks able to respond appropriately to complex combinations of extracellular signals.

Curiously, in one respect the mathematical formalism of artificial neural networks is a more accurate approximation for networks of protein molecules than for networks of real neurons. The processing of electrical signals by a nerve cell is considerably more complex than the static sigmoidal nonlinearity of individual allosteric proteins. Many other features of biochemical signalling pathways, however, have no counterpart in most conventional computer-based neural nets³⁸. Thus the architecture of the cytoplasmic network lacks well-defined boundaries, and individual units are not all equivalent in performance. The time-scales of interactions between 'units' also varies enormously.

Arguably, the most important defining characteristic of protein-based neural networks is that they are governed by diffusive processes. Signals pass by means of physical contact between molecules, and their dispersion through the cytoplasm is limited by the random thermal motion of molecules. For small molecules and ions, diffusion through a cell is rapid: a molecule such as cyclic AMP can reach any part of a mammalian cell in a tenth of a second. Proteins are larger and diffuse more slowly, and are often impeded in their progress by associations with other components. Indeed the crowded conditions within a living cell force many proteins together in associations not seen *in vitro*³⁹. Many steps in signal transduction consequently take place between protein molecules that are in physical contact, moving rapidly through the multimeric structure ('signalosome') by means of propagated allosteric changes or by internal catalytic changes. In the chemotactic relay system of coliform bacteria discussed previously, for example, a complex of proteins that includes CheA, CheW and CheY is associated with the cytoplasmic domain of individual membrane receptors⁴⁰. Such complexes evidently work as computational cassettes that produce a stereotypical response to a specific input. Note that, because the links between individual protein molecules in such oligomeric assemblies can be modified by evolution, proteins are still the fundamental 'units' of computation from a neural network standpoint. It is just that these units are clustered spatially and limited in the extent of connections they make to other modules: signalling between signalosomes is presumably mediated by freely diffusing elements, especially second messengers of low molecular mass. □

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- Stryer, L. *Biochemistry* (Freeman, San Francisco, 1995).
- Johnson, L. N. & Barford, D. A. *Rev. Biophys. struct. Biol.* **22**, 199–232 (1994).
- Hanson, P. I., Meyer, T., Stryer, L. & Schuilman, H. *Neuron* **12**, 943–956 (1994).
- Anwyl, R. *Curr. Biol.* **4**, 854–856 (1994).
- Pawson, T. & Schlessinger, J. *Curr. Biol.* **3**, 434–442 (1993).
- Saitiel, A. R. *FASEB J.* **8**, 1034–1040 (1994).
- Morgan, D. O., Kaplan, J. M., Bishop, J. M. & Varmus, H. E. *Cell* **57**, 775–786 (1989).
- Roach, P. J. *FASEB J.* **4**, 2961–2968 (1990).
- Hille, B. *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA, 1992).
- McCulloch, W. S. & Pitts, W. *Bull. math. Biophys.* **5**, 115–133 (1943).
- Minsky, M. & Papert, S. *Perceptrons* (MIT Press, Cambridge, MA, 1969).
- Rumelhart, D. E., McClelland, J. L. *Parallel Distributed Processing. Explorations in the Microstructure of Cognition* (MIT Press, Boston, MA, 1986).
- Arkin, A. & Ross, J. *Biophys. J.* **67**, 560–578 (1994).
- Shacter, E., Chock, P. B. & Stadtman, E. R. *J. Biol. Chem.* **259**, 12252–12259 (1984).
- Stadtman, E. R. & Chock, P. B. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2761–2765 (1977).
- Goldbeter, A. & Koshland, D. E. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6840–6844 (1981).
- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **346**, 125–132 (1990).
- Hepler, J. R. & Gilman, A. G. *Trends biochem. Sci.* **17**, 383–387 (1992).
- Boguski, M. S. & McCormick, F. *Nature* **366**, 643–654 (1993).
- Hall, A. *Science* **264**, 1413–1414 (1994).
- Gilman, A. G. A. *Rev. Biochem.* **56**, 615–649 (1987).
- Chock, P. B. & Stadtman, E. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2766–2770 (1977).
- Okamoto, M., Sakai, T. & Hayashi, K. *Biol. Cybern.* **58**, 295–299 (1988).
- Hjelmfelt, A., Schneider, F. W. & Ross, J. *Science* **260**, 335–337 (1993).
- Bray, D. & Lay, S. *Biophys. J.* **66**, 972–977 (1994).
- Koshland, D. E., Goldbeter, A. & Stock, J. B. *Science* **217**, 220–225 (1982).
- Lamb, T. D. & Pugh, E. N. *J. Physiol. Lond.* **449**, 710–758 (1992).
- Stryer, L. *Cold Spring Harb. Symp. quant. Biol.* **53**, 283–294 (1988).
- Bray, D., Bourret, R. B. & Simon, M. I. *Molec. Biol. Cell* **4**, 469–482 (1993).
- Parkinson, J. S. & Kofoid, E. C. A. *Rev. Genet.* **26**, 71–112 (1992).
- Berg, H. C. & Tedesco, P. M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3235–3239 (1975).
- Lin, D. M. & Goodman, C. S. *Neuron* **13**, 507–523 (1994).
- Kishimoto, T., Taga, T. & Akira, S. *Cell* **76**, 253–262 (1994).
- Tjian, R. & Maniatis, T. *Cell* **77**, 5–8 (1994).
- Anholt, R. R. H. *Trends Neurosci.* **17**, 37–41 (1994).
- Murray, A. & Hunt, T. *The Cell Cycle* (Oxford Univ. Press, Oxford, 1993).
- Bray, D. *Cell Movements* (Garland Publishing, New York, 1992).
- Bray, D. *J. theor. Biol.* **143**, 215–231 (1990).
- Zimmerman, S. B. & Minton, A. P. A. *Rev. Biophys. struct. Biol.* **22**, 27–65 (1994).
- Gegner, J. A., Graham, D. R., Roth, A. F. & Dahlquist, F. W. *Cell* **70**, 975–982 (1992).

Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex

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The crystal structure of the human cyclinA-cyclin-dependent kinase2 (CDK2)-ATP complex has been determined at 2.3 Å resolution. CyclinA binds to one side of CDK2's catalytic cleft, inducing large conformational changes in its PSTAIRE helix and T-loop. These changes activate the kinase by realigning active site residues and relieving the steric blockade at the entrance of the catalytic cleft.

THE eukaryotic cell cycle is coordinated by several related Ser/Thr protein kinases, each consisting of a catalytic cyclin-dependent kinase (CDK) subunit and a regulatory cyclin subunit (reviewed in refs 1, 2). The transient appearance of these cyclin-CDK complexes drives cell cycle events such as cell growth³ (cyclinD-CDK4 and cyclinD-CDK6 in G1), DNA replication^{4,5} (cyclinE-CDK2 in G1/S and cyclinA-CDK2 in S) and cell division⁶ (cyclinA-CDK1 and cyclinB-CDK1 in G2 and M).

The CDK subunit is inactive as a protein kinase without the cyclin subunit⁷. The binding of the cyclin subunit confers basal kinase activity to the complex⁸, and phosphorylation of a threonine residue in the CDK subunit by CDK-activating kinase⁷ (CAK) results in full activity. Recent studies indicate that binding of the cyclin subunit also modulates the substrate specificity of the kinase⁹. Substrates include the retinoblastoma protein, Rb, which is preferentially associated with cyclinD-CDK4 complexes¹⁰; p107 (ref. 11), E2F (ref. 12), and replication protein-A (ref. 13), which are phosphorylated by cyclinA-CDK2; and histone H1, which is phosphorylated by cyclinB-CDK1 (refs 13, 14).

Cyclins, which vary in relative molecular mass (M_r) from 30K to 45K, share a homologous region of about 100 amino acids (30–50% similarity among cyclins A, B, D1 and E) termed the cyclin box¹⁵. CDKs are highly homologous⁹ (40–75% similarity among CDK1 through CDK7) and contain a conserved catalytic core of approximately 300 amino acids common to all eukaryotic protein kinases¹⁶. Crystallographic studies of several eukaryotic protein kinases^{17–20}, including CDK2 (ref. 21) and the cyclic AMP-dependent protein kinase catalytic subunit (PKA)²² have shown that they all share the same fold and tertiary structure. When these structures are compared in detail, however, significant differences in their catalytic sites become evident, which are thought to reflect differences in their regulation. Whereas CDKs are activated by the binding of their regulatory cyclin subunit, for example, PKA is active in the free form and the binding of its regulatory subunit is inhibitory²².

To understand how cyclins activate CDKs, we have determined the crystal structure of a human cyclinA-CDK2-ATP complex at 2.3 Å resolution and refined it to a crystallographic R -factor of 20.8%. Comparison of the CDK2 structure in this complex with that of catalytically inactive free CDK2 (ref. 21) reveals that cyclin binding causes large conformational changes in CDK2's active site which we propose are responsible for activating CDKs.

Structure determination

Because human cyclinA aggregates at high concentrations, we used limited proteolytic digestion to identify a 29K structural

domain more amenable to crystallization (residues 173–432). This fragment, which contains core sequences common to members of the cyclin family, has CDK2 binding and activating properties identical to those of full-length cyclinA. The histone H1 phosphorylation activity of the CDK2-cyclinA fragment complex is 0.30 pmol phosphate transferred per min per pmol CDK2, compared to 0.28 for the CDK2-full-length cyclinA complex. Addition of a CDK-activating kinase preparation increases the activities to 5.1 and 4.8 pmol phosphate, respectively. Similar results have been obtained with truncated *Xenopus laevis* cyclinA fragments analogous to the one reported here^{23,24}. Both complexes are inhibited to the same extent by the p27^{Kip1} CDK inhibitor protein²⁵ (0.024 residual activity), and both cyclinA molecules have similar *in vivo* mitotic activity when synthesized in *Xenopus laevis* oocytes from microinjected capped complementary RNA²⁴.

The cyclinA fragment-CDK2-ATP complex produced three crystal forms from similar crystallization conditions (Table 1). An orthorhombic form in spacegroup $P2_12_12_1$ with $a=132.9$, $b=149.3$, $c=73.7$ Å grew readily; a hexagonal form in spacegroup $P6_222$ with $a=b=185.1$, $c=214.4$ Å grew infrequently; and a third form grew twinned and was not characterized. Both characterized forms contain two complexes in the asymmetric unit and have similar non-crystallographic symmetry (approximate two-fold symmetry). The structure of the complex in the orthorhombic crystals was determined by multiple isomorphous replacement (Table 1), and the structure of the complex in the hexagonal form was determined by molecular replacement using the partially refined orthorhombic form structure (Table 1). The structures in the two forms are essentially identical, and here we consider the hexagonal form, which has been refined at 2.3 Å resolution. The current model of the asymmetric unit contains 2 cyclinA molecules (residues 173–432), 2 CDK2 molecules (residues 1–298), 2 ATP molecules and 416 water molecules. The structure (Fig. 1) has an R -factor of 20.8% for data from 6.0 to 2.3 Å, with 0.013 Å r.m.s. deviation from ideal bond lengths and 1.77° r.m.s. deviation from ideal bond angles.

Overall structure of the complex

The structure of CDK2 consists of an amino-terminal lobe (residues 1–85) rich in β -sheet and a larger, mostly α -helical, carboxy-terminal lobe. ATP binds in a deep cleft between the two lobes which contains catalytic residues conserved among eukaryotic protein kinases and is presumed to be the site of protein substrate binding and catalysis. CyclinA has two helical domains with identical chain topology. It binds to one side of the catalytic cleft, interacting with both lobes of CDK2 to form a large, continuous protein-protein interface (Fig. 2a, b)

TABLE 1 Diffraction data, MIR analysis and refinement statistics

Data set	Resolution (Å)	Reflections		Data coverage (%)	R_{sym} (%)	MIR analysis (20–3.2 Å)		
		Measured	Unique			Sites	MFID (%)†	Phasing power‡
Native 2 (P6 ₂ 22)	2.3	677,413	86,466	90.2	6.1			
Native-Mg (P2 ₁ 2 ₁ 2 ₁)	2.5	174,498	47,667	92.3	6.5			
Native 1 (P2 ₁ 2 ₁ 2 ₁)	3.1	81,900	26,362	96.3	4.6			
HgCl ₂	3.2	63,110	23,344	93.3	4.4	2	0.12	2.56
Thimerosal	3.4	35,140	20,928	78.8	5.9	5	0.17	1.31
UO ₂ (OAc) ₂	3.2	75,102	24,218	96.0	5.9	2	0.16	1.62
Sm(OAc) ₃	3.4	41,907	17,685	85.1	6.6	2	0.12	0.43
K ₂ Au(CN) ₄	3.8	31,511	13,104	86.7	7.4	6	0.11	0.81
r.m.s.								
Refinement	Resolution (Å)	Reflections (with $ F > 2\sigma$)	Total number of atoms	waters	R -factor (%)§	Bonds (Å)	Angles (°)	B-factor (Å ²)
Hexagonal	6.0–2.3	70,208	9,476	416	20.8	0.013	1.77	3.0
Orthorhombic	7.0–2.5	43,576	9,157	97	22.8	0.013	1.89	2.5

Protein expression and purification. Human cyclinA was expressed in *Escherichia coli* and purified as described⁸. The cyclinA fragment (residues 173–432) was produced by digesting 50 mg cyclinA with 25 µg subtilisin at 4 °C for 90 min, and it was purified by cation exchange chromatography (the identity of the fragment was determined by N-terminal sequencing and by subcloning into *E. coli*). The N-terminal 172 residues absent in the fragment do not have significant homology in other cyclins, and are absent from cyclins D and E¹⁵. Human CDK2 was expressed in insect cells using a baculovirus vector, and was purified as described³⁴. Mass spectroscopy (by the electrospray ionization triple-stage quadrupole method) of the baculovirus-expressed CDK2 preparation indicated that it consisted predominantly of a single species (>95%). The mass was consistent with the unphosphorylated CDK2 molecule having an acetyl group at the N-terminal methionine (Edman degradation negative). The complex was prepared by mixing CDK2 and the cyclinA fragment at an equivalent molar ratio, followed by purification on a gel filtration column and concentration by ultrafiltration to 25 mg ml⁻¹ in a buffer of 40 mM HEPES, 0.2 M NaCl, 5 mM dithiothreitol (DTT), pH 7.0. **Crystallization and data collection.** Crystals were grown at 4 °C by the hanging drop vapour diffusion method. The complex was mixed with an equal volume of the well buffer containing 28% saturated (NH₄)₂(SO₄), 1 M KCl, 40 mM HEPES, 5 mM DTT, pH 7.0, and ATP was added to the drop at a final concentration of 10 mM. Magnesium was not used in the crystallization because it interfered with the growth of large, diffraction-quality crystals. All diffraction data were collected at -170 °C. The Native-1 and derivative data sets were collected using an R-AxisIIIC imaging plate detector mounted on a Rigaku 200HB generator. The Native-2 and Native-Mg data sets were collected using a CCD detector at the A1 beamline of the Cornell High Energy Synchrotron Source (MacChess). For flash freezing, crystals were successively transferred to cryoprotectant buffers that contained increasing amounts of glycerol (10, 20 and 30%), and were then flash frozen in a stream of nitrogen gas at -170 °C in 50% saturated (NH₄)₂(SO₄), 30% glycerol, 50 mM KCl, 5 mM ATP, 100 mM HEPES, pH 7.0. **MIR analysis, model building and refinement.** Heavy-atom soaks were performed using the orthorhombic form crystals in a buffer of 50% saturated (NH₄)₂(SO₄), 0.25 M KCl, 5 mM ATP and 100 mM HEPES, pH 7.0, containing one of the following heavy-atom solutions: 2 mM HgCl₂ for 5 h, 0.6 mM thimerosal for 15 h, 4 mM uranyl acetate for 5 days, 3 mM samarium acetate for 5 h, or 5 mM dipotassium gold cyanide for 13 h. Initial MIR phases were calculated at 3.2 Å resolution with the program PHARE³⁵ and had a mean figure of merit of 0.62. The MIR map was improved by solvent flattening³⁶ and non-crystallographic symmetry (n.c.s.) averaging with the program RAVE³⁷. The initial MIR map and the n.c.s.-averaged map were used to build the initial model with the programs O³⁸ and CHAIN³⁹ (CDK2 was built based on a model derived from the PKA structure). Successive rounds of rebuilding, simulated annealing refinement with the program X-PLOR⁴⁰, and phase combination with the program SIGMAA³⁵ allowed complete interpretation of the cyclinA and CDK2 structures in the orthorhombic crystal form. After several cycles of refinement with the full model, X-PLOR omit maps were used to systematically check every part of the complex (about 2–5% of the structure was deleted in each calculation, and simulated annealing was used to reduce model bias in the omit maps). **Molecular replacement solution of the hexagonal form structure.** The structure of the hexagonal form was determined by molecular replacement with the programs MERLOT⁴¹, BRUTE⁴² and X-PLOR using a monomeric cyclinA-CDK2 model from the orthorhombic form crystal structure. The molecular replacement search yielded two distinct solutions which corresponded to the same n.c.s.-related dimer observed in the orthorhombic crystal form. The molecular replacement model was refined by simulated annealing (X-PLOR). Successive rounds of rebuilding and refinement were followed by restrained temperature factor refinement and anisotropic temperature factor correction (X-PLOR). $2F_o - F_c$ and $F_o - F_c$ electron density maps in the hexagonal crystal form had clear electron density for the adenine ring, the sugar, and the α -phosphate group of ATP which was consistent with the MIR electron density observed with the orthorhombic crystal form. In the final stages, ATP and water molecules were added to the model. The model was then further refined by simulated annealing with X-PLOR, and least-squares refinement with TNT⁴³. The β - and γ -phosphate groups of the ATP and the glycine-rich loop of CDK2 (residues 11–16) that anchors the phosphate groups have poor electron density in the maps, and high temperature factors in the refined model (similar apparent flexibility in the glycine-rich loop and phosphate groups have been observed in other kinase structures¹⁷). Other regions with high temperature factors and weak electron density include the 20 C-terminal amino acids of cyclinA (after the second repeat), and residues 38–41 of CDK2. **Structure in the presence of magnesium.** Because magnesium interfered with crystal growth, we attempted to determine whether this was caused by magnesium altering the structure of the bound ATP or of the cyclinA-CDK2 complex. An orthorhombic form cyclinA-CDK2-ATP crystal was soaked in 4 mM MgCl₂ for 2 days (Native-Mg) without any effect on diffraction quality. The 2.5 Å refined structure of the cyclinA-CDK2-ATP-Mg complex indicates that there are no significant changes in the structure when magnesium is present. The position of the magnesium was verified using data from a cyclinA-CDK2-ATP crystal soaked in a samarium solution [Sm(OAc)₃-Derivative]. The electron-dense samarium atom was located unambiguously in the catalytic site with difference Patterson and difference Fourier methods; it is bound by Asp 145 and has a position similar to that of the activating magnesium atom in the PKA structure²².

* $R_{\text{sym}} = \sum_n \sum_i |I_{h,i} - I_h| / \sum_n \sum_i I_{h,i}$ for the intensity (I) of i observations of reflection h .

† MFID, mean isomorphous difference = $\sum |F_{\text{PH}} - F_P| / \sum F_{\text{PH}}$, where F_{PH} and F_P are the derivative and native structure factors, respectively.

‡ Phasing power = $[(F_{\text{H(c)}})^2 / (F_{\text{PH(o)}} - F_{\text{PH(c)}})^2]^{1/2}$.

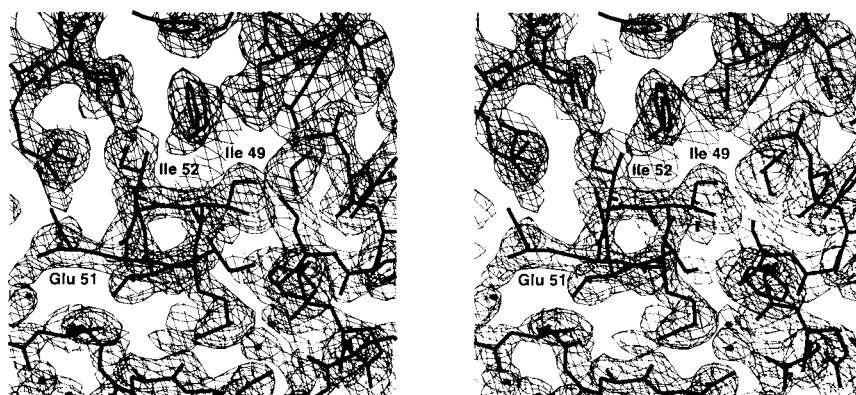
§ R -factor = $\sum |F_o - F_c| / \sum F_o$.

|| Root mean square deviations from ideal geometry, and r.m.s. variation in the B-factor of bonded atoms.

The $\alpha 1$ helix of CDK2, which contains the PSTAIRE sequence motif characteristic of the cyclin-dependent kinase family⁹ (Fig. 2a–c), is central to the interface. In cyclinA-bound CDK2, this helix rotates about its axis and moves several ångströms into the catalytic cleft, compared to free CDK2 (ref. 21). To make

space for it, the N-terminal sheet of CDK2 tilts away. The T-loop of CDK2, which contains the site phosphorylated in the fully active kinase (Thr 160 (ref. 26)), also interacts with cyclinA (Fig. 2a, b). Compared to free CDK2, this loop undergoes large conformational and positional changes which

FIG. 1 Electron density at the cyclinA-CDK2 interface in the vicinity of the PSTAIRE helix contoured at 1.2σ . The $(2|F_o| - |F_c|)$ Fourier synthesis was calculated at 2.3 Å resolution using phases calculated after omitting the interface residues shown, and subjecting the model to simulated annealing refinement (X-PLOR) from 2500K (interface residues within 12 Å of Ile 49 were omitted). Ile 49, Glu 51 and Ile 52 are labelled.



alter the positions of its residues by as much as 21 Å. CyclinA also contacts the N-terminal β -sheet and helices in the C-terminal lobe of CDK2.

Structure of cyclinA

The cyclinA fragment has a globular structure consisting of 12 α -helices. The molecule contains a structural repetition, having two sequential 90 amino acid domains with identical folds (residues 208–303 and 309–399, respectively; Fig. 3a, b). These folds consist of a right-handed three-helix bundle ($\alpha 1$, $\alpha 2$ and $\alpha 3$) with two additional helices ($\alpha 4$ and $\alpha 5$) packed against the bundle's side (the second repeat helices are named $\alpha 1'$ to $\alpha 5'$). The very similar structure of the two repeats—the C α atoms of 78 of the 90 amino acids can be superimposed with an r.m.s. deviation of 1.7 Å (Fig. 3b)—was unexpected as the repeats are only 12% identical (Fig. 3c).

The repeats are connected by a linker of five amino acids, and pack loosely against each other across an interface that contains

three buried water molecules. They can be superimposed by a 160° rotation and an 11 Å translation about an axis approximately parallel to the helix bundle axis. In addition to the 10 helices in the two repeats, there is an additional α -helix (residues 179–190) N-terminal to the first repeat that packs against the second repeat, a helical extension to the second repeat (residues 408–412) and an extended region at the C terminus of the protein (residues 413–432).

The first repeat coincides with the cyclin box (residues 209–310; Fig. 3c). As expected from its sequence conservation, it is the key element at the cyclin-CDK2 interface, forming the binding site for the PSTAIRE helix and making contacts with the T-loop and the N-terminal β -sheet of CDK2. In general, the conservation of the different cyclinA elements across species parallels their role in CDK2 binding. For example, the first repeat is 78% similar to clam cyclinA²⁷; the N-terminal helix, which interacts with the C-terminal lobe of CDK2 and makes a large contribution to the buried surface area, is 56% similar;

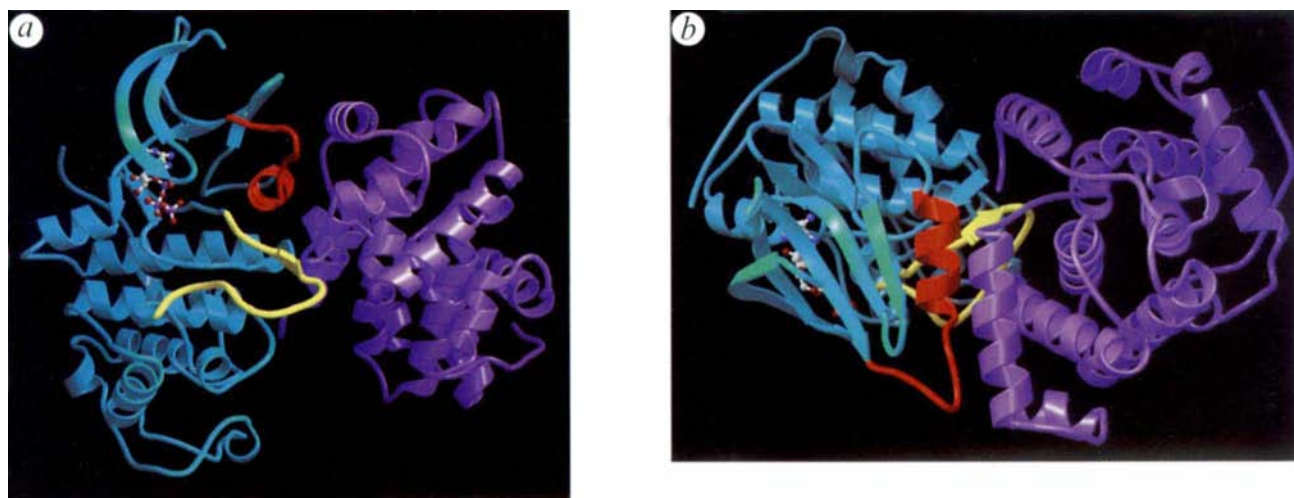
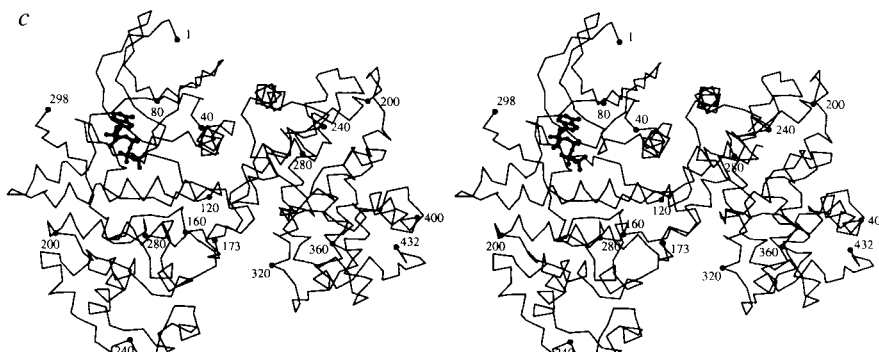


FIG. 2 a, Schematic drawing of the cyclinA-CDK2 complex. CyclinA is coloured magenta, CDK2 light blue; the ATP is shown as a ball and stick representation. Portions of CDK2 that undergo large conformational changes upon cyclinA binding are highlighted: the PSTAIRE region of CDK2 is red, the T-loop of CDK2 yellow. b, View of the complex looking down the vertical axis of a. The figures were done with the programs MOLSCRIPT⁴⁴ and RASTER3D⁴⁵. c, Stereo view of the C α trace of the cyclinA-CDK2 complex in an orientation similar to that of Fig. 2a. Approximately every 40th residue is numbered and its C α atom highlighted as a circle. The ATP is shown as a ball and stick representation.



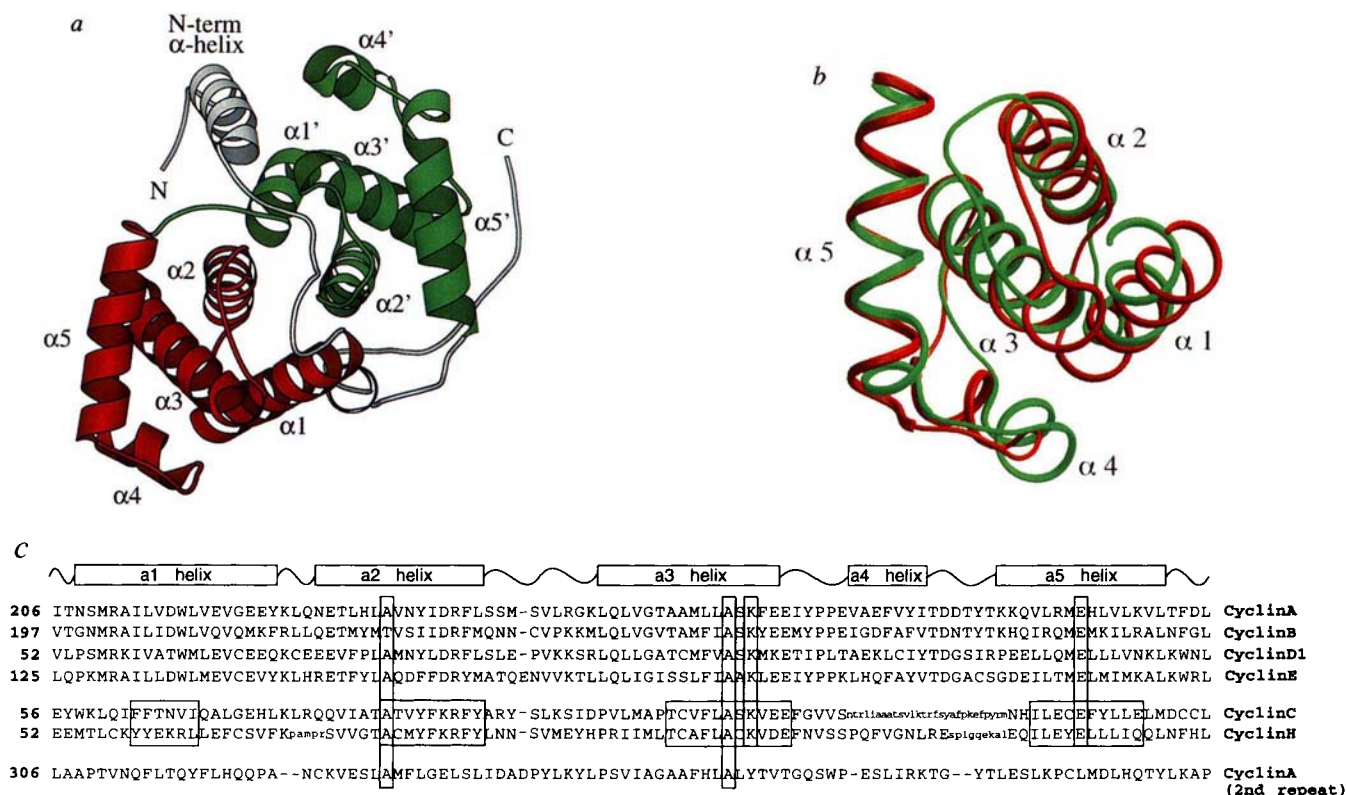


FIG. 3 **a**, View of the cyclinA structure (residues 173–432) highlighting the two structural repeats. The first repeat is coloured red, the second repeat green, the N- and C-terminal helices grey, and the individual helices are labelled. The two repeats are related by a 160° rotation and an 11 Å translation about an axis perpendicular to the plane of the figure positioned approximately between helices $\alpha 2$ and $\alpha 2'$. The orientation of cyclinA is similar to that of Fig. 2b. **b**, The two repeats have very similar structures, and their C α atoms can be superimposed with r.m.s. deviation of 1.7 Å (for 78 of the 90 amino acids). The backbone atoms for residues 211–303 of the first repeat (red) and residues 311–399 of the second repeat (green) are represented schematically. The different orientation of the $\alpha 1$ helix in the first repeat is correlated with a buried salt bridge between Arg 211 of the $\alpha 1$ helix and Asp 240 of the $\alpha 2$ helix. The salt bridge is buttressed by hydrogen bonds from the Arg 211 guanidinium group to the hydroxyl of Tyr 199 and to the backbone carbonyl groups of Leu 341 and Ile 342 (from the second repeat). The second repeat does not have an equivalent salt bridge, and its $\alpha 1'$ helix thus packs closer to the structure. **c**, The first cyclin

fold repeat of cyclinA coincides with the cyclin box, a region of approximately 100 amino acids conserved among members of the cyclin family. Sequence alignment showing the cyclin boxes from cyclinB (50% similarity to the cyclin box of cyclinA), cyclinD1 (40% similarity) and cyclinE (40% similarity) aligned with that of cyclinA¹⁵. Cyclins C²⁸ and H⁷ are more distantly related to cyclinA. The second cyclin fold repeat of cyclinA was aligned according to the structural alignment in **b**. The vertical boxes highlight the conserved alanine residues, which are likely to be characteristic of the cyclin fold, and also the lysine and glutamic acid residues that contact the backbone of CDK2 (the second cyclinA repeat does not contact CDK2 and thus it does not have the conserved lysine and glutamic acid residues). The boxes around the cyclin C²⁸ and H⁷ sequences indicate stretches of hydrophobic residues that match similar residues in the cyclin box cyclins. Insertions in the cyclin C and H sequences are in lower-case letters and probably replace the $\alpha 4$ helix. The secondary structure elements of cyclinA, indicated above the sequence, were defined according to criteria published by Kabsch and Sander⁴⁶.

and the second repeat, which plays only a minor role in the interface, is 48% similar.

Structure of the cyclin fold. The structural motif of five helices observed twice in the cyclinA fragment is probably common to other members of the cyclin family, and may be termed the cyclin fold. The hydrophobic core of the motif is formed by the $\alpha 3$ helix (Fig. 3a, b) whose central portion is surrounded by the remaining four helices. The $\alpha 1$ and $\alpha 2$ helices, which pack against the $\alpha 3$ helix at 43° and –47°, respectively, participate both in forming the hydrophobic core and in the inter-repeat packing with helices $\alpha 1'$ and $\alpha 2'$. Helices $\alpha 4$ and $\alpha 5$, which pack against the $\alpha 3$ helix at 39° and 105°, respectively, are partially exposed to solvent and contact CDK2. Similar interhelical angles occur in the second repeat.

The large angles between helices are associated with short interhelical distances where they cross. These crossing points involve alanine residues, whose small size allows the helices to pack tightly. The most significant of these packing interactions involves alanines 235 and 264 from the $\alpha 2$ and $\alpha 3$ helices, respectively (equivalent to alanines 333 and 363 from the second repeat), where the tight packing allows no substitutions.

This pair of residues is highly conserved among cyclin family members, including cyclins C and H which are only distantly related to the cyclin box cyclins^{7,28} (Fig. 3c). Additional crossing-point residues with small side chains include Gly 257, Ala 259 and Ala 260 from the $\alpha 3$ helix (equivalent to Ala 356, Ala 358 and Ala 359 from the second repeat). Although not as well conserved, these compact residues are also present in members of the cyclin family (Fig. 3c). This pattern of compact amino acids may thus be characteristic of the cyclin fold.

Structure of the cyclinA–CDK2 interface

The cyclinA–CDK2 interface is formed from an interlocking array of CDK2 and cyclinA elements (Fig. 4a), including the PSTAIRE helix, the T-loop, portions of the N-terminal sheet and C-terminal lobe from CDK2, and helices $\alpha 3$, $\alpha 4$ and $\alpha 5$ from the first repeat, as well as the N-terminal helix from cyclinA (Fig. 4a, b). Although the density of side chain contacts is not uniform, being highest around the PSTAIRE helix and lowest at the N-terminal sheet and C-terminal lobe, they result in a large buried surface area of 3,550 Å² (Fig. 4a, b).

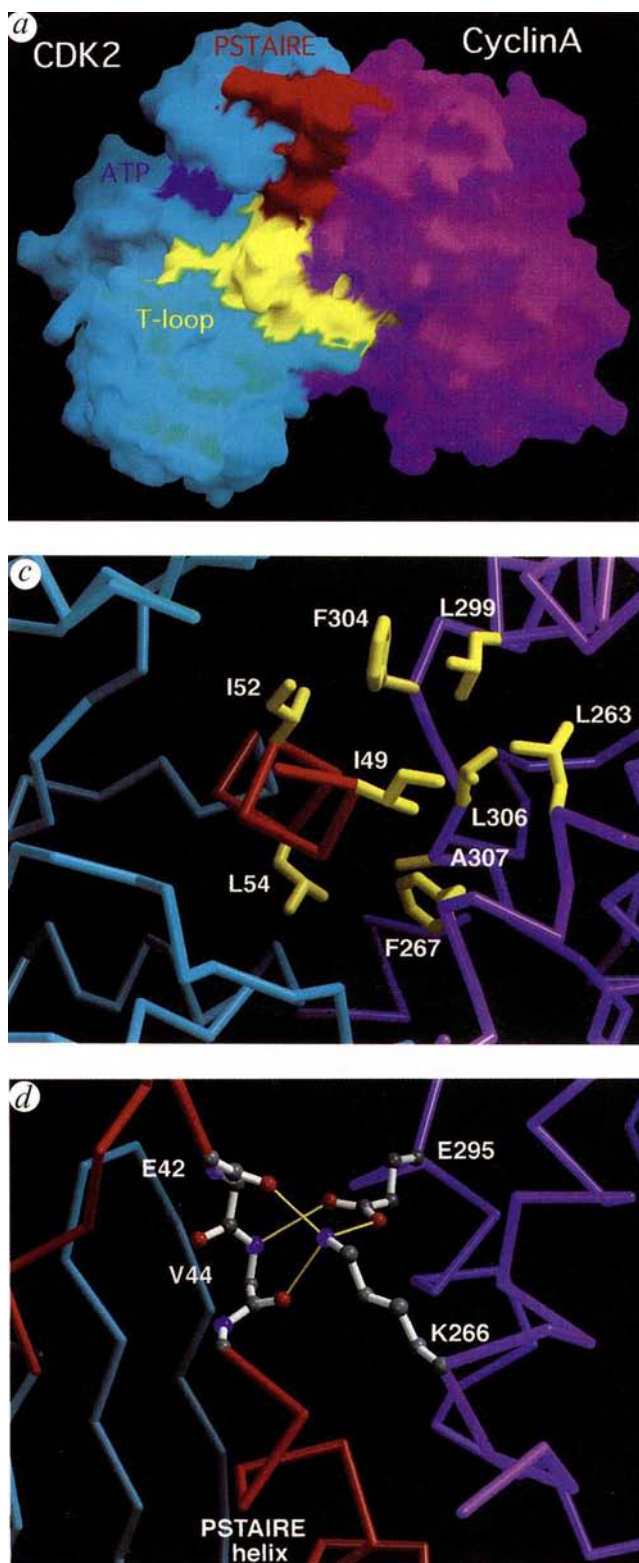
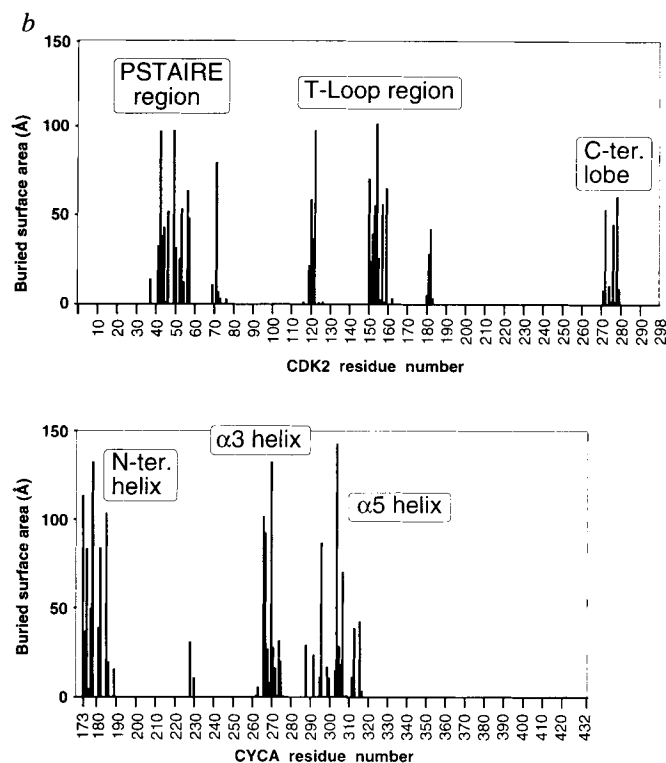


FIG. 4 *a*, The cyclinA-CDK2 interface consists of interdigitated cyclinA and CDK2 structural elements. Surface representation of the cyclinA (magenta)-CDK2 (cyan) complex highlighting the PSTAIRE region in red and the T-loop in yellow. The solvent-exposed ATP surface is shown

PSTAIRE region of CDK2. As shown in Fig. 2*a*, the cyclin box clamps onto and surrounds the middle part of the PSTAIRE helix (residues 46–56): the $\alpha 5$ helix lies parallel to the PSTAIRE helix on one side, and the C terminus of the $\alpha 3$ helix abuts the other side perpendicularly. This part of the PSTAIRE helix is



blue (orientation similar to that of Fig. 2*a*). The figure was done with the program GRASP⁴⁷. The surface area buried upon complex formation is substantially larger than most other heterologous protein-protein complexes whose structure is known, including the complex of enterotoxin B with a class II major histocompatibility molecule⁴⁸ (1540 Å² buried), and antibody-protein complexes⁴⁹ (typically 1500 Å² buried). *b*, Bar graph showing the differences in solvent accessibility⁵⁰ in cyclinA and CDK2 residues in the complex compared to the isolated subunits. The approximate locations of the structural elements discussed in the text are indicated. The cyclinA-CDK2 interface contains approximately 25 hydrophobic residues whose solvent accessibility is reduced substantially upon complex formation, and also contains 17 intermolecular hydrogen bonds between polar or charged amino acids. The following cyclinA-CDK2 interactions are not mentioned in the text: in the N-terminal β -sheet of CDK2, Val 69, His 71, Leu 76 and Leu 37 pack against His 296 and Phe 304 of cyclinA; in the C-terminal lobe, His 121 and the aliphatic portion of the Arg 122 side chain (both from the $\alpha 3$ helix of CDK2) make van der Waals contacts with Tyr 185 and Ile 182 of cyclinA, respectively, whereas the backbone amides of Ser 277 and Ala 278 (from the $\alpha 7$ helix of CDK2) donate a pair of hydrogen bonds to the hydroxyl of Tyr 178 of cyclinA. *c*, Close-up *Ca* view of the interface showing the extensive hydrophobic side chain (yellow) interactions between the PSTAIRE helix of CDK2 and the cyclin box of cyclinA in an orientation and colouring similar to that of Fig. 2*a*. To make the buried hydrophobic interactions easier to see, only a section of the interface along the direction of the view axis is shown. Ile 49, Ile 52 and Leu 54 of CDK2, and Leu 263, Phe 267, Leu 299, Leu 306 and Ala 307 of cyclinA are labelled. In the structure of free CDK2, Ile 49 and Ile 52 of the PSTAIRE helix point away from the solvent and would be mostly inaccessible for cyclinA binding. *d*, Close up *Ca* view of the network of side chain-backbone hydrogen-bond interactions in the loop preceding the PSTAIRE helix. To make the interactions easier to see, only the Lys 266 and Glu 295 side chains of cyclinA and the backbone groups of Glu 42–Val 44 of CDK2 are shown. Colours are as Fig. 2*a* except that atoms of interacting residues are red (oxygen), blue (nitrogen) and grey (carbon). The view is slightly rotated about the x- and y-axes with respect to that of Fig. 2*a*.

thus bound by an extended patch of hydrophobic interactions (Fig. 4*c*), whereas the N- and C-terminal regions are bound by networks of hydrogen bonds. Ile 49, in particular, fits tightly into a hydrophobic pocket lined with the side chains of Leu 263, Phe 267, Leu 299, Leu 306 and the aliphatic portion of Lys 266

from cyclinA (Fig. 4c). In addition, Ile 52 makes a van der Waals contact with Phe 304 of cyclinA and Leu 54 packs against Phe 267 and Ala 307 of cyclinA (Fig. 4c).

Immediately preceding the PSTAIRE helix, the backbone carbonyl groups of Glu 42 and Val 44 accept two hydrogen bonds from the Lys 266 side chain of cyclinA, and the backbone amide of Val 44 donates a hydrogen bond to the Glu 295 carboxylate of cyclinA (Fig. 4d). An additional hydrogen bond links the Lys 266 and Glu 295 side chains (Fig. 4d). The CDK2 loop conformation that allows this hydrogen-bond network relies on Gly 43, which adopts a backbone conformation ($\phi = 112^\circ$, $\psi = -150^\circ$) that other amino acids cannot adopt. The critical residues in this interaction, Lys 266 and Glu 295 of cyclinA, and Gly 43 of CDK2, are highly conserved.

In the C-terminal portion of the PSTAIRE helix, Lys 56 of CDK2 donates two hydrogen bonds to the Asp 305 side chain and the Thr 303 backbone carbonyl of cyclinA, and Glu 57 of CDK2 accepts a hydrogen bond from the Tyr 185 hydroxyl group of cyclinA.

The key role of the PSTAIRE helix at the interface is consistent with existing biochemical and genetic data. The exact sequence in this region correlates with the cyclin preference of CDKs⁹, and mutations there eliminate cyclin binding^{29,30}. Furthermore, the binding of anti-PSTAIRE antibodies is blocked by cyclins³¹.

The T-loop of CDK2. The T-loop region of CDK2 (residues 146–166), which blocks the entrance to the catalytic cleft in the free

kinase, is another major focus of cyclinA binding. In the complex, the N-terminal portion of the T-loop (residues 150–157) is bound by helices $\alpha 1$, $\alpha 2$ and $\alpha 3$ from the first repeat of cyclinA, as well as its N-terminal helix (Fig. 4a, b). Significant interactions include van der Waals contacts between Ala 151, Phe 152 and Tyr 159 of CDK2, and Phe 267, Ile 182 and Ile 270 of cyclinA, respectively. Hydrogen bonds also link the Arg 150 guanidinium group of CDK2 to the Glu 269 and Ile 270 backbone carbonyl groups of cyclinA and the side chains of Arg 157 (CDK2) and Gln 228 (cyclinA). CyclinA binding induces large conformational and positional changes in the T-loop, significantly relieving the blockade of the catalytic cleft observed in free CDK2.

N-terminal sheet and C-terminal lobe of CDK2. Interactions between the N-terminal β -sheet of CDK2 and the $\alpha 5$ helix of cyclinA are predominantly hydrophobic, whereas those between the C-terminal lobe of CDK2 and the N-terminal helix of cyclinA are also polar (Fig. 4a, b). Although these interactions are not as extensive as those of the PSTAIRE helix, they effectively exclude solvent and thus substantially extend the area of buried interface (Fig. 4a, b), presumably stabilizing the complex.

Conformational changes in CDK2

CyclinA alters the conformation of CDK2's PSTAIRE region and T-loop, and the relative orientation of its N- and C-terminal lobes. The C-terminal lobe, with the exception of the T-loop, is

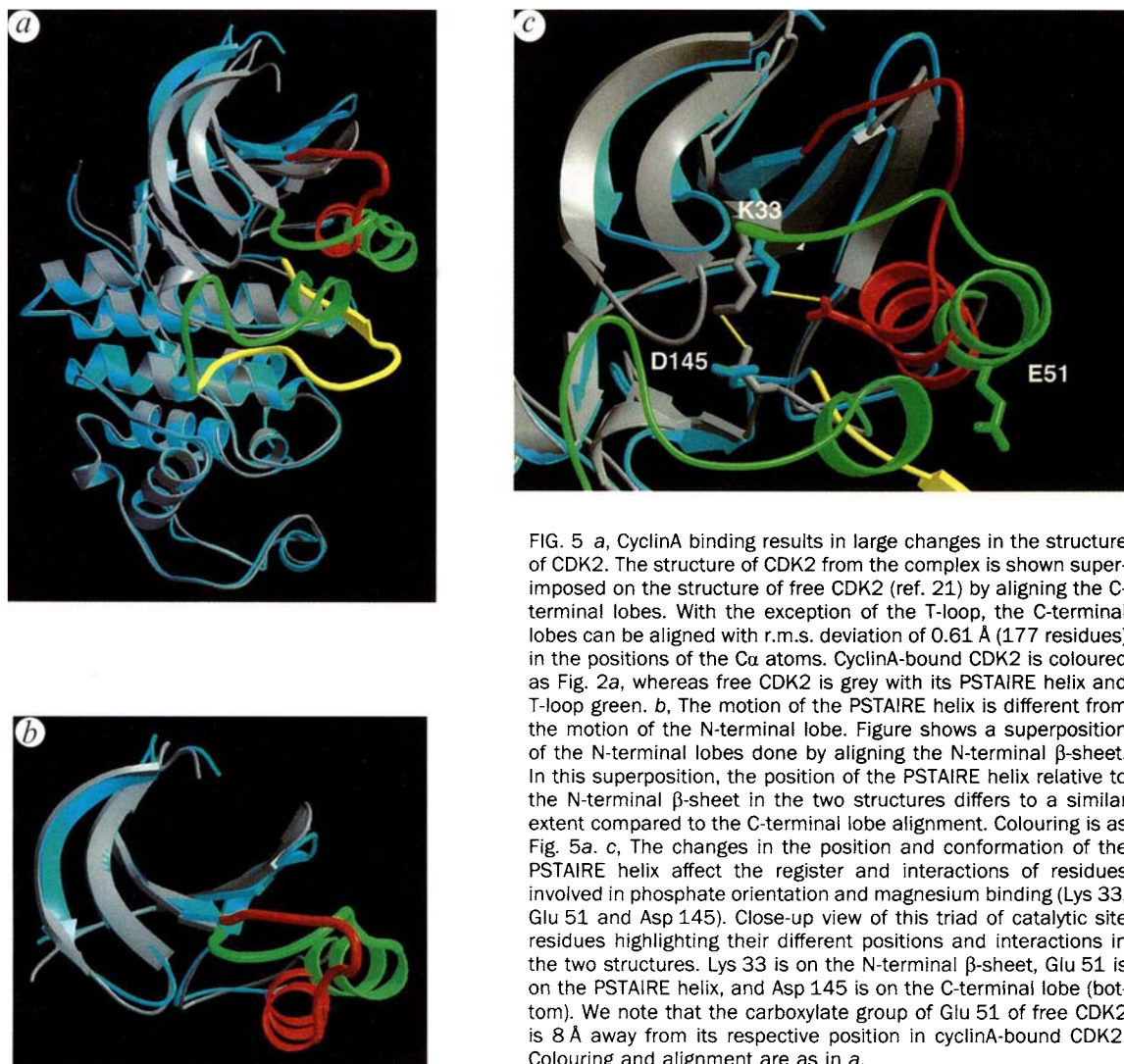


FIG. 5 a, CyclinA binding results in large changes in the structure of CDK2. The structure of CDK2 from the complex is shown superimposed on the structure of free CDK2 (ref. 21) by aligning the C-terminal lobes. With the exception of the T-loop, the C-terminal lobes can be aligned with r.m.s. deviation of 0.61 Å (177 residues) in the positions of the C α atoms. CyclinA-bound CDK2 is coloured as Fig. 2a, whereas free CDK2 is grey with its PSTAIRE helix and T-loop green. b, The motion of the PSTAIRE helix is different from the motion of the N-terminal lobe. Figure shows a superposition of the N-terminal lobes done by aligning the N-terminal β -sheet. In this superposition, the position of the PSTAIRE helix relative to the N-terminal β -sheet in the two structures differs to a similar extent compared to the C-terminal lobe alignment. Colouring is as Fig. 5a. c, The changes in the position and conformation of the PSTAIRE helix affect the register and interactions of residues involved in phosphate orientation and magnesium binding (Lys 33, Glu 51 and Asp 145). Close-up view of this triad of catalytic site residues highlighting their different positions and interactions in the two structures. Lys 33 is on the N-terminal β -sheet, Glu 51 is on the PSTAIRE helix, and Asp 145 is on the C-terminal lobe (bottom). We note that the carboxylate group of Glu 51 of free CDK2 is 8 Å away from its respective position in cyclinA-bound CDK2. Colouring and alignment are as in a.

not significantly affected, and can be superimposed on that of free CDK2 with a 0.61-Å r.m.s. deviation in the Ca positions (Fig. 5a). The C-terminal lobe will thus serve as a frame of reference in discussing the changes in CDK2.

Changes in the PSTAIRE region. The PSTAIRE helix starts with a helical hydrogen bond from the conserved Pro 45. On binding cyclinA, extensive hydrogen bonding (Fig. 4c) induces a conformational change in the loop immediately preceding the PSTAIRE helix (Fig. 5a, b), moving the Pro 45 starting residue. This is associated with a translation of the helix into the catalytic cleft towards the ATP and a rotation of roughly 90° about its helical axis, so that the backbone atoms in the helix are as much as 8.5 Å away from their counterparts in free CDK2 (Fig. 5a, b). The rotation changes the relative 'phase' of the helices in the two structures by approximately one residue. Towards the C terminus of the helix, the differences become smaller and the structures converge, as the C terminus of the helix is overwound and has a 3_0 -like conformation in free CDK2.

The altered position and orientation of the PSTAIRE helix are associated with dramatic changes in its packing against the rest of CDK2. In free CDK2, packing is only loose, and the helix is apparently positioned to facilitate the conformational change accompanying cyclinA binding. Only two CDK2 residues make significant packing contacts: Ala 48 against the α L12 helix; and Ile 52 against the N-terminal lobe.

In cyclinA-bound CDK2, however, packing is much tighter, owing to (1) the lateral translation and the rotation of the PSTAIRE helix, and (2) the melting of the α L12 helix at the

beginning of the T-loop which prevents motion of the PSTAIRE helix in free CDK2. In the complex, the repositioned Ala 48 and Ile 52 pack extensively with Ile 35, Val 69, Leu 76 and Leu 78 in the N-terminal lobe. Furthermore, Leu 54, which in free CDK2 pointed out into solution, now points towards the C-terminal lobe and packs with Val 123 and Ala 151, while Leu 55, which was pointing into a water-filled internal cavity, now packs against Phe 80 and Phe 146.

The significance of this movement is that it brings the side chain of Glu 51, which belongs to a triad of catalytic site residues conserved in all eukaryotic kinases¹⁶, into the catalytic site. This triad (Lys 33, Glu 51 and Asp 145) is involved in ATP phosphate orientation and magnesium coordination, and is thought to be critical for catalysis³². In cyclinA-bound CDK2, Glu 51 lies in the catalytic cleft and makes a salt bridge with Lys 33, one of the residues that anchors the α and β phosphate groups of ATP, whereas Asp 145 binds the magnesium atom (Fig. 5c). These interactions are essentially identical to those observed in the catalytically active PKA structure²². In free CDK2, however, Glu 51 is outside the catalytic cleft and is mostly exposed to solvent. Its carboxylate group lies 8 Å from its position in cyclinA-bound CDK2, and cannot therefore form a salt bridge with Lys 33 (Fig. 5c). Instead, Lys 33 makes a salt bridge with Asp 145 (ref. 21), resulting in these residues adopting conformations and functions that differ from those observed in cyclinA-bound CDK2 and in PKA (Fig. 5c). This misalignment of active site residues probably plays a large part in the catalytic inactivity of free CDK2 (ref. 16).

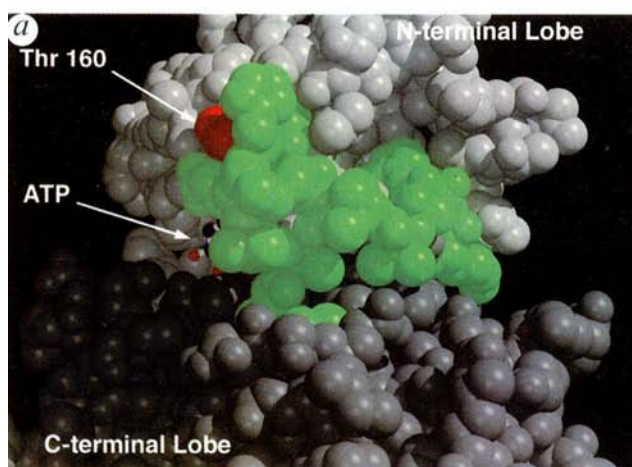
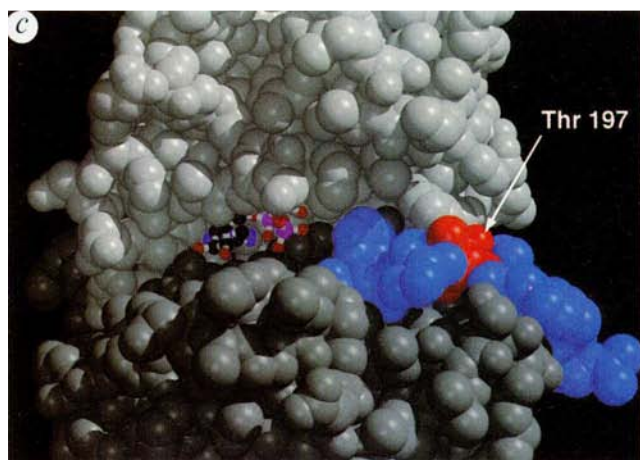
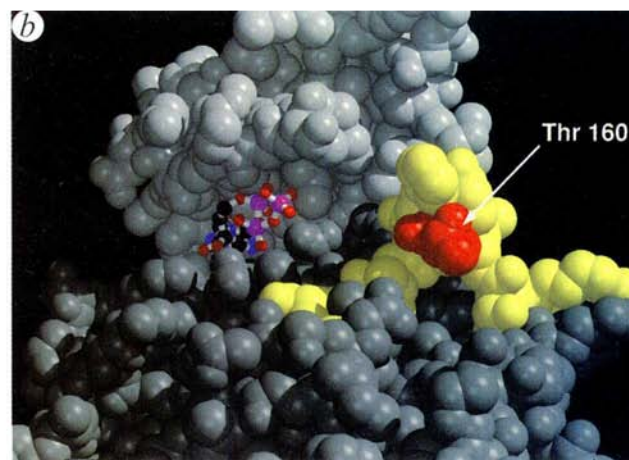


FIG. 6 a, CPK model highlighting the T-loop position relative to the ATP in the structure of free CDK2 (ref. 21). The C-terminal lobe is coloured dark grey, the N-terminal lobe light grey, the T-loop green and the Thr 160 phosphorylation site red. The ATP is mostly buried behind the



T-loop in this orientation. This conformation of the T-loop is presumed to block access of the protein substrate to the catalytic site²¹. b, CPK model highlighting the T-loop in the structure of cyclinA-bound CDK2. The colouring is as a, except that the T-loop is yellow (the structure was aligned on that of free CDK2 by superimposing the C-terminal lobes). The conformational change in the T-loop significantly relieves the blockade of the catalytic site by making the γ -phosphate of ATP fully accessible to the protein substrate. The change in the T-loop also exposes the Thr 160 side chain (red), making it a better substrate for phosphorylation. c, CPK model highlighting the catalytic site in the structure of the PKA (ref. 22). The region corresponding to the T-loop is shown blue, and the Thr 197 phosphorylation site red (the structure was aligned on that of free CDK2 by superimposing the C-terminal lobes). In cyclinA-bound CDK2 (b) and PKA (c), part of the loop is to the right and back side of the cleft and is thus not visible in this view. The conformational changes the T-loop undergoes upon binding to cyclinA make it resemble closely the respective region of PKA. With the exception of three residues at the Thr 160 phosphorylation site of cyclinA-bound CDK2, the T-loop (residues 146–166) can be superimposed on the equivalent region of PKA (residues 185–202) with r.m.s. deviation of 1.65 Å in the positions of their Ca atoms. It is only in the immediate vicinity of the phosphorylation site that the two loops differ significantly where the Ca atom of Thr 160 is 5 Å away from the Ca atom of Thr 197, the equivalent residue in PKA.

Changes in the T-loop. In free CDK2, the Gly 147–Gly 153 region of the T-loop forms the α L12 helix, packing with the PSTAIRE helix and the N-terminal lobe (Fig. 5a). In cyclinA-bound CDK2, the α L12 helix melts, allowing the region instead to form a reverse turn (residues 146–149), followed by a β -sheet (residues 150–152) with the β 6 strand (residues 122–124) of the C-terminal lobe (Fig. 5a; a similar β -sheet occurs in the catalytically active PKA structure). The change also leads to new packing interactions with the repositioned PSTAIRE helix and with cyclinA (Arg 150, Ala 151 and Phe 152 interact with cyclinA as discussed earlier).

This change has a twofold significance. First, the α L12 helix prevents the PSTAIRE helix from moving into the catalytic cleft in free CDK2, and its melting on binding cyclinA enables the PSTAIRE helix to move. Second, the β -sheet that replaces the α L12 helix directs the T-loop away from the entrance of the catalytic cleft. At the end of the β -sheet, the T-loop is already 15 Å away from its position in free CDK2.

Beyond the α L12 region, the T-loop in free CDK2 (residues 154–164) criss-crosses the entrance of the catalytic cleft, rendering the ATP inaccessible to the protein substrate (Fig. 6a). In cyclinA-bound CDK2, this region is 12–21 Å from its position in free CDK2, well away from the entrance of the catalytic cleft and close to cyclinA (Fig. 6b; Arg 157 and Tyr 159 make additional contacts with cyclinA, as discussed earlier). The cyclinA-induced detour ends at the 164–166 region, by the end of which the Ca atoms in the bound and free forms are within 0.5 Å of each other. This detour substantially relieves the steric hinderance at the entrance to the catalytic cleft (Fig. 6a, b) and exposes the Thr 160 hydroxyl group phosphorylated by CAK, which in free CDK2 is buried in the catalytic cleft²¹ (Fig. 6a, b).

Comparison of the Thr 160 phosphorylation site with that of PKA. The conformational and positional changes induced by cyclinA in the T-loop leave it closely resembling the respective region of PKA²² (Fig. 6b, c). In cyclinA-bound CDK2, however, the Thr 160 side chain is unphosphorylated and points out into solution (Fig. 6b), whereas in PKA the equivalent residue (Thr 197) is phosphorylated, and its side chain is held close to the structure by multiple salt bridges between the phosphate group and three basic side chains (Fig. 6c). In the PKA–substrate complex²², this region forms part of the binding pocket for the hydrophobic substrate residue immediately following the phosphate acceptor, and its different conformation in the

unphosphorylated cyclinA-bound CDK2 may explain why the latter is only partially active.

When CDK2 is phosphorylated, the Thr 160 region may adopt a conformation similar to that of PKA, as cyclinA-bound CDK2 contains a cationic pocket that might bind the Thr 160 phosphate group in a manner analogous to that of PKA. The pocket contains the side chains of Arg 50, Arg 126 and Arg 150, which bind the carboxylate of Glu 162 in the unphosphorylated complex.

Change in the relative orientation of the N- and C-terminal lobes of CDK2. Apart from the PSTAIRE region, the isolated N-terminal lobe is not significantly altered by cyclin binding (Fig. 5b), but it moves relative to the C-terminal lobe, tilting away from the PSTAIRE helix and the entrance of the catalytic cleft so as to open the cleft (Fig. 5a). The overall rotation is about 14°, pivoting about a hinge region behind the cleft.

This movement makes space for the PSTAIRE helix to move into the catalytic cleft, but is otherwise unconnected with the motion of the PSTAIRE helix (Fig. 5b). The N-terminal lobe movement is also coupled to the changes the T-loop undergoes. In free CDK2, the T-loop criss-crosses the entrance to the catalytic cleft and interacts with the N-terminal β -sheet. On binding cyclinA, however, the N-terminal lobe's movement would not allow this interaction even if the T-loop did not move towards cyclinA. Finally, the N-terminal lobe movement also brings the side chain of Tyr 15, whose phosphorylation is thought to inhibit the kinase³³, deeper into the catalytic cleft. In the complex, the Tyr 15 side chain is within hydrogen-bonding distance of the Glu 51 carboxylate, and its phosphorylation may affect the positions of catalytic site residues and the ATP phosphates through steric hindrance and electrostatic repulsion.

Conclusion

The crystal structure of the cyclinA–CDK2 complex offers a general model for cyclin binding and CDK activation. Intimate interactions between the PSTAIRE region and the cyclin box form the centrepiece of the cyclin–CDK interface, and are likely to be key determinants of cyclin–CDK specificity. The activation of the kinase results from a conformational change in the PSTAIRE helix that realigns active site residues, together with a large motion of the T-loop that relieves the blockade of the catalytic cleft. The cyclin–T-loop interactions also expose Thr 160, making it a better substrate for phosphorylation by CAK and setting the stage for the full activation of the kinase. □

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- Morgan, D. O. *Nature* **374**, 131–134 (1995).
- Pines, J. *Biochem. Soc. Trans.* **21**, 921–925 (1993).
- Sherr, C. J. *Cell* **79**, 551–555 (1994).
- Heichman, K. A. & Roberts, J. M. *Cell* **79**, 557–562 (1994).
- Sobczak-Thépot, J. et al. *Exp. Cell Res.* **206**, 43–48 (1993).
- King, R. W., Jackson, P. K. & Kirschner, M. W. *Cell* **79**, 563–571 (1994).
- Fisher, R. P. & Morgan, D. O. *Cell* **78**, 713–724 (1994).
- Connell-Crowley, L., Solomon, M. J., Wei, N. & Harper, J. W. *Molec. Biol. Cell* **4**, 79–92 (1993).
- Pines, J. *Semin. Cancer Biol.* **5**, 305–313 (1994).
- Ewen, M. E. et al. *Cell* **73**, 487–497 (1993).
- Peeper, D. S. et al. *EMBO J.* **12**, 1947–1954 (1993).
- Dynlacht, B. D., Flores, O., Lees, J. A. & Harlow, E. *Genes Dev.* **8**, 1772–1786 (1994).
- Nigg, E. A. *Curr. Opin. Cell Biol.* **5**, 187–193 (1993).
- Langan, T. A. et al. *Molec. cell. Biol.* **9**, 3860–3868 (1989).
- Hadwiger, J. A., Wittenberg, C., Richardson, H. E., De Barros Lopes, M. & Reed, S. I. *Proc. natn. Acad. Sci. U.S.A.* **86**, 6255–6259 (1989).
- Taylor, S. S. & Radzio-Andzelm, E. *Structure* **2**, 345–355 (1994).
- Zhang, F., Strand, A., Robbins, D., Cobb, M. H. & Goldsmith, E. J. *Nature* **367**, 704–711 (1994).
- Hubbard, S. R., Wei, L., Ellis, L. & Hendrickson, W. A. *Nature* **372**, 746–754 (1994).
- Hu, S. H. et al. *Nature* **369**, 581–584 (1994).
- Xu, R. M., Carmel, G., Sweet, R. M., Kuret, J. & Cheng, X. *EMBO J.* **14**, 1015–1023 (1995).
- De Bondt, H. L. et al. *Nature* **363**, 595–602 (1993).
- Knighton, D. R. et al. *Science* **253**, 407–413 (1991).
- Kobayashi, H. et al. *Molec. Biol. Cell* **3**, 1279–1294 (1992).
- Lees, E. M. & Harlow, E. *Molec. cell. Biol.* **13**, 1194–1201 (1993).
- Polyak, K. et al. *Cell* **78**, 59–66 (1994).
- Desai, D., Gu, Y. & Morgan, D. O. *Molec. Biol. Cell* **3**, 571–582 (1992).
- Standart, N., Dale, M., Stewart, E. & Hunt, T. *Genes Dev.* **4**, 1257–1268 (1990).
- Lew, D. J., Dulic, V. & Reed, S. I. *Cell* **66**, 1197–1206 (1991).
- Ducummun, B. et al. *EMBO J.* **10**, 3311–3319 (1991).

- Marcote, M. J. et al. *Molec. cell. Biol.* **13**, 5122–5131 (1993).
- Pines, J. & Hunter, T. *Cell* **58**, 833–846 (1989).
- Zheng, J. et al. *Biochemistry* **32**, 2154–2161 (1993).
- Solomon, M. J. *Curr. Opin. Cell Biol.* **5**, 180–186 (1993).
- Rosenblatt, J., De Bondt, H., Jancarik, J., Morgan, D. O. & Kim, S. J. *molec. Biol.* **230**, 1317–1319 (1993).
- SERC (UK) Collaborative Computing Project No. 4 (Daresbury Laboratory, Warrington, 1979).
- Furey, W. & Swaminathan, S. *Am. Crystallogr. Ass. Meet. Abstr. Ser.* **2**, 18, 73 (1990).
- Jones, T. A. in *Molecular Replacement* (ed. Dodson, E. J.) 91–105 (SERC, Daresbury, 1992).
- Jones, T. A., Zou, J.-Y. & Cowan, S. W. *Acta crystallogr.* **A47**, 110 (1991).
- Sack, J. S. J. *molec. Graphics* **6**, 224 (1988).
- Brunker, A. T. X-PLOR, a system for crystallography and NMR, Version 3.0 (Yale Univ. Press, New Haven, CT, 1991).
- Fitzgerald, P. M. D. J. *appl. Crystallogr.* **21**, 273 (1988).
- Fujinaga, M. & Read, R. J. *appl. Crystallogr.* **20**, 273 (1987).
- Tonrud, D. E., Ten Eyck, L. F. & Matthews, B. W. *Acta crystallogr.* **A43**, 489 (1987).
- Kraulis, P. J. *appl. Crystallogr.* **24**, 946–950 (1991).
- Merritt, E. A. & Murphy, M. E. *Acta crystallogr.* **D50**, 869–873 (1994).
- Kabsch, W. & Sander, C. *Biopolymers* **22**, 2577–2637 (1983).
- Nicholls, A., Sharp, K. A. & Honig, B. *Proteins: Structure, Function and Genetics*, **11**, 281–296 (1991).
- Jardetzky, T. S. et al. *Nature* **368**, 711–718 (1994).
- Davies, D. R., Padlan, E. A. & Sheriff, S. A. *Rev. Biochem.* **59**, 439–473 (1990).
- Lee, B. & Richards, F. M. J. *molec. Biol.* **55**, 379–400 (1971).

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Extensive dark-matter haloes in low-luminosity galaxies revealed by quasar absorption lines

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STUDIES of the kinematics of spiral galaxies suggest that dark (possibly non-baryonic) matter is a significant component of their total mass¹. It has been difficult to determine how far the dark-matter haloes of galaxies extend, however, because the presence of dark matter must be inferred from the motion of a luminous component such as atomic gas, which becomes hard to detect beyond several galactic radii. The recent suggestion² that the Lyman- α absorption lines seen in the spectra of distant quasars arise in the extended gaseous haloes of intervening galaxies offers an alternative means of probing their dark-matter content. Here we present observations of two low-luminosity spiral galaxies which have redshifts coincident with those of Lyman- α absorption lines in the spectra of quasars that are near the galaxies in the sky. The gas responsible for the absorption lines appears to take part in the rotation of the galaxies themselves, suggesting that the haloes of both galaxies are dominated by dark matter which remains a significant component of the haloes out to significantly greater radii than previously demonstrated.

To get accurate redshifts of the absorbing galaxies and to test their kinematics out to the largest possible radii, new optical spectra of two absorbing galaxies close on the sky to the bright quasars 1704+6048 and 2135-1446 were obtained with the spectrograph slit aligned to the apparent major axis of the galaxy. The extracted spectra had a resolution of 35–40 km s⁻¹ and were reduced to vacuum, heliocentric wavelengths. Accurate mean galaxy redshifts are given in Table 1.

Ultraviolet spectra of the background quasars 1704+6048 and 2135-1446, obtained by the Hubble Space Telescope (HST) using the Faint Object Spectrograph (FOS) and G130H grating and accessed via the HST archive, reveal absorption systems which coincide in velocity with the galaxy redshifts. Because the absolute wavelength calibration of FOS spectra can have large systematic errors, heliocentric redshifts for the absorbers (Table 1) were derived following procedures described in ref. 3.

Optical R-band images of the galaxies towards 1704+6048 and 2135-1446 were used to obtain estimates for the galaxy inclination and orientation angles and radial scale lengths. Model exponential disk surface-brightness profiles were fitted to the images using χ^2 fitting routines. Both galaxies have exponential disk surface-brightness profiles, with little or no evidence for central bulges (see Table 1 for exponential disk scales). Figure 1 shows a portion of the image around 2135-1446.

The observations described above can be combined and used to derive rotation curves of the galaxies. First, we consider the intrinsic rotation curves, that is, the rotation curves in the disk planes of the galaxies. The intrinsic rotation curves trace the underlying potential wells of the galaxies. Assuming circular motions, the intrinsic rotation curves $v_{\text{int}}(R)$ are obtained from the observed rotation curves $v_{\text{obs}}(R)$ by using the relation

$$v_{\text{int}}(R) = v_{\text{obs}}(R)/\sin i$$

where i is the galaxy inclination angle and R is the galactocentric radius. The results are shown in Fig. 2.

From Fig. 2 it is seen that the rotation curve of the galaxy towards 1704+6048 is approximately flat beyond a galacto-

FIG. 1 R-band CCD image of the field around quasar 2135-1446; see Table 1. The image is 90 × 90 arcsec and the seeing was ~1 arcsec.

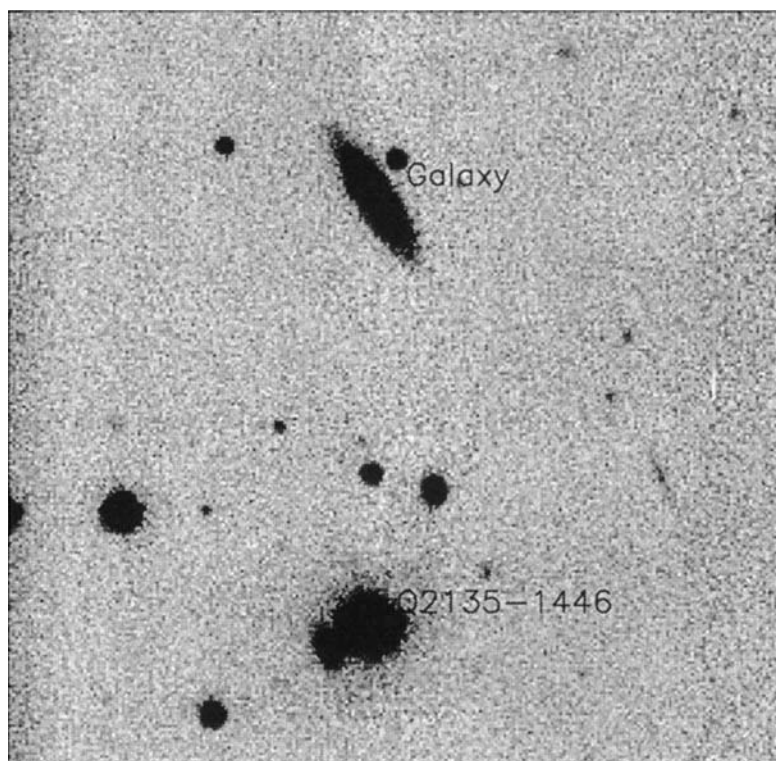


TABLE 1 Properties of the galaxies and absorbers towards quasars 1704+6068 and 2135-1446

	1704+6048	2135-1446
Absorber redshift	0.09204 ± 0.00005	0.07506 ± 0.00013
Absorption impact parameter ρ (h^{-1} kpc)	62	48
Galaxy RA (1950)	17 h 03 min 56.6 s	21 h 35 min 01.1 s
Galaxy dec. (1950)	+60° 48' 49"	-14° 45' 37"
Galaxy offset from quasar	50.9" W, 18.2" N	0.7" W, 49.4" N
Galaxy redshift	0.09163 ± 0.00002	0.07578 ± 0.00005
Galaxy disk inclination (degrees)	69 ± 1	72 ± 0.5
Galaxy disk major axis PA (degrees)	162	32
Galaxy radial scale length (h^{-1} kpc)	1.77 ± 0.17	2.93 ± 0.25
Galaxy $M_B - 5 \log h$	-17.3	-17.7
Galaxy $B - V$	0.6	0.6
Galaxy mass $R < 10 h^{-1}$ kpc ($10^{10} M_\odot$)	2.8 ± 0.4	5.2 ± 0.5
Galaxy mass $R < \rho$ ($10^{10} M_\odot$)	$>10 \pm 3$	$>23 \pm 8$

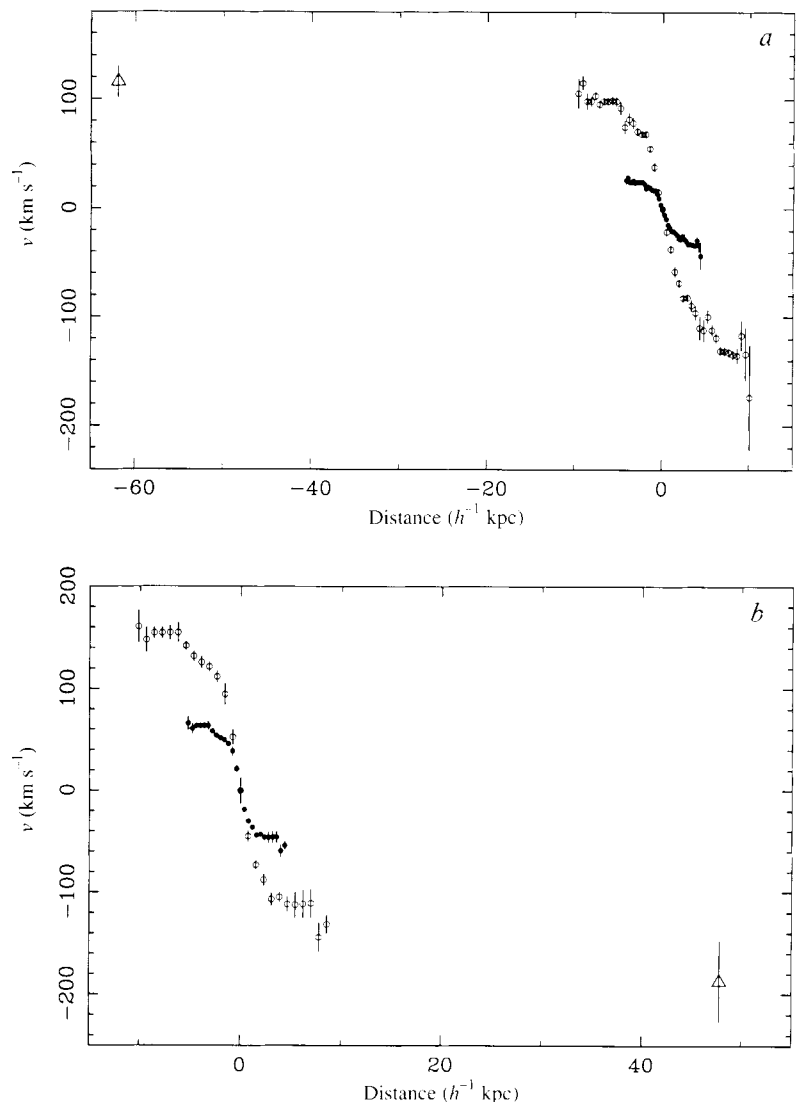
Galaxy positions, inclinations, position angles, scale lengths and photometry are derived from CCD (charge-coupled device) images. The image around 1704+6048 was obtained with the Isaac Newton Telescope at the Observatorio del Roque de los Muchachos (Canary Islands, Spain)²; the image around 2135-1446 was obtained with the New Technology Telescope.

centric radius of $\sim 5 h^{-1}$ kpc (where h is the Hubble constant H_0 in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$) with a rotation velocity of $\sim 120 \text{ km s}^{-1}$; the rotation curve of the galaxy towards 2135-1446 is approximately flat beyond a galactocentric radius of $\sim 5 h^{-1}$ kpc with a rotation velocity of $\sim 150 \text{ km s}^{-1}$. The rotation velocities of the galaxies are significantly less than the rotation velocities ($v_{\text{int}} \approx 220 \text{ km s}^{-1}$) of normal, luminous spiral galaxies, suggesting that the galaxies are underluminous. Specifically, applying the Tully-Fisher relationship to the observed rotation velocities of the galaxies, the luminosity of the galaxy towards 1704+6048 implied by the intrinsic rotation curve is $\sim 0.09 L_*$ and that of the galaxy towards 2135-1446 is $\sim 0.22 L_*$, L_* being the typical B-band luminosity of a luminous spiral galaxy corresponding to an absolute magnitude of $M_B = -19.5$.

The luminosities implied by the intrinsic rotation curves can be compared with the luminosities derived from photometric observations by adopting the observed-frame R-band absolute magnitudes and convolving the low-resolution spectra of the galaxies with the approximate filter response functions. The derived rest-frame absolute B-band magnitudes and $B - V$ colours of the absorbing galaxies are given in Table 1; they are consistent with the luminosities implied by the intrinsic rotation curves. The $B - V$ colours of both galaxies are similar to those of spiral galaxies ($B - V < 0.7$)⁴.

The two galaxies studied here have surface-brightness profiles, radial scale lengths, rotation velocities⁵ and luminosities con-

FIG. 2 Galaxy rotation curves and velocity differences between galaxies and absorbers. a, The galaxy and absorber towards quasar 1704+6048; b, the galaxy and absorber towards quasar 2135-1446. Open circles, intrinsic rotation curves $v_{\text{int}}(R)$ of the galaxies, where R is the galactocentric radius; solid circles, projected rotation curves $v_{\text{pro}}(\rho)$ of the galaxies, where ρ is the projected impact parameter; open triangles, velocity differences between the galaxies and the absorbers as a function of ρ . Error bars give 1σ uncertainties derived from gaussian profile fits to the H α emission line. The spectra were obtained with the ISIS spectrograph on the William Herschel Telescope at the Observatorio del Roque de los Muchachos (Canary Islands, Spain).



sistent with those of extreme late-type intermediate to dwarf galaxies. We therefore conclude that both galaxies are low-luminosity late-type spiral galaxies. The H α rotation curves demonstrate that, out to the galactocentric distance at which the line is detected ($\sim 10 h^{-1}$ kpc in both cases), the dynamics of both galaxies are dominated by unseen matter, with estimated mass-to-light ratios of 20 and $30 h (M_{\odot}/L_{\odot})$ for the absorbing galaxies towards 1704+6048 and 2135–1446 respectively, consistent with values obtained in other low-luminosity spiral galaxies^{6,7}.

Second, we consider the projected rotation curves, that is, the rotation curves projected onto the lines of sight along the projected line segments joining the galaxies to the quasars. The projected rotation curves trace the galaxy motions appropriate for comparison with the velocity differences between the galaxies and the absorbers. Assuming circular motions, the projected rotation curves ($v_{\text{pro}}(\rho)$, where ρ is the projected impact parameter; see below) are obtained from the intrinsic rotation curves $v_{\text{int}}(R)$ by using the relation

$$v_{\text{pro}}(\rho) = v_{\text{int}}(R) \cos \alpha \sin i (1 + \sin^2 \alpha \tan^2 i)^{-1/2}$$

where α is the angle between the apparent major axis of the galaxy and the projected line segment joining the galaxy to the quasar and $\rho = R(1 + \sin^2 \alpha \tan^2 i)^{-1/2}$. The results are also shown in Fig. 2, together with the velocity differences between the galaxies and the absorbers as functions of projected impact parameter.

From Fig. 2 it is seen that the velocity differences between the galaxies and the absorbers match extrapolations of the projected rotation curves in sense but lie significantly above these extrapolations. This is contrary to expectation, assuming circular motion and that the absorption arises in unwarped gaseous extensions of the stellar disks. To illustrate this further, we have computed the circular rotation velocity needed for the galaxy disks to produce the projected velocity shift at the point where the quasar light is absorbed. These circular velocities would be $360 \pm 44 \text{ km s}^{-1}$ and $-456 \pm 95 \text{ km s}^{-1}$ at disk radii $R = 93 h^{-1}$ kpc and $R = 92 h^{-1}$ kpc respectively for the galaxies towards 1704+6048 and 2135–1446, that is much too large for the measured potential wells. A shallow increase of rotation velocity with radius has been reported previously for Sb and Sc galaxies⁸ ($v_{\text{int}}(R) \propto R^{\beta}$, with $\beta \approx 0.1\text{--}0.2$), but to explain our observations with extended gaseous disks would require a steep increase of rotation velocity with radius ($\beta \approx 1\text{--}1.5$). We therefore conclude that the absorption does not arise in gaseous extensions of the stellar disks.

From Fig. 2 it is also seen that the velocity differences between the galaxies and their corresponding Lyman- α absorbers are in striking agreement with the extrapolations of the intrinsic rotation curves of the galaxies in both sense and magnitude. This suggests that the absorbing material—which occurs at galactocentric distances far greater than the stellar disks traced by the rotation curves, see Fig. 1—takes part in the galactic rotation. Hence the absorption might arise in gaseous haloes co-rotating with the stellar disks. Although difficult to understand physically, the present observations could be explained if the absorbing material is distributed around the galaxies in geometrically thick, co-rotating structures with vertical dimensions of $\sim 40 h^{-1}$ kpc. In any case, these data indicate either that typical velocities in the haloes are slightly larger than in the disks (so that the projected velocities of the absorbing clouds coincide with the intrinsic rotation curves) or that, by chance, the velocities of the clouds are in both cases approximately aligned with the line of sight. We cannot distinguish between these possibilities with a sample of only two objects, so more definite conclusions must await the study of a larger sample.

Under the assumption that the absorbing material is gravitationally bound to the galaxies, the lower limit to the dynamical mass of the galaxy towards 1704+6048 is $M > (1.0 \pm 0.3) \times 10^{11} M_{\odot}$, and the lower limit to the dynamical mass of the galaxy towards 2135–1446 is $M > (2.3 \pm 0.8) \times 10^{11} M_{\odot}$, leading to

lower limits to the mass-to-light ratios of $M/L > 77(M/L)_{\odot}$ and $M/L > 124(M/L)_{\odot}$ respectively, within the distances where Lyman- α absorption occurs. Similar values have been obtained previously for dwarf spiral galaxies^{6,7} (from studies of the 21-cm emission line) but within much smaller radii. Our results demonstrate that quasar absorption systems can be used to trace the dynamics of the absorbing galaxies at very large radii.

We find it intriguing that the first two galaxy absorption-system pairs examined in detail involve low-luminosity spiral galaxies. Apparently, at least some extreme late-type galaxies possess substantial gaseous cross-sections at the moderate column densities traced by Lyman- α absorption whereas such galaxies do not possess significant gaseous cross-sections at the higher column densities traced by Mg II absorption systems⁹.

Our study demonstrates that low-luminosity galaxies possess haloes which are dark-matter-dominated out to very large radii. Lyman- α absorption lines can be detected in HST spectra down to column densities of $10^{14}\text{--}10^{15} \text{ cm}^{-2}$ (that is, several orders of magnitude below any reasonable detection threshold for a 21-cm emission line) and therefore can be used to probe gaseous haloes out to large galactocentric distances. This technique is particularly suitable for the study of low-luminosity spiral galaxies. $\square$

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1. van Albada, T. S. & Sancisi, R. *Phil. Trans. R. Soc.* **320**, 15–32 (1986).
2. Lanzetta, K. M., Bowen, D. V., Tytler, D. & Webb, J. K. *Astrophys. J.* **442**, 538–568 (1995).
3. Bahcall, J. N. et al. *Astrophys. J. Suppl. Ser.* **87**, 1–43 (1993).
4. Mihalas, D. & Binney, J. *Galactic Astronomy* 2nd edn 356 (Freeman, San Francisco, 1981).
5. Casertano, S. & van Gorkom, J. H. *Astr. J.* **101**, 1231–1241 (1991).
6. Carigan, C. & Freeman, K. C. *Astrophys. J.* **332**, L33–L36 (1988).
7. Lake, G., Schommer, R. A. & van Gorkom, J. H. *Astr. J.* **99**, 547–560 (1990).
8. Rubin, V. in *Internal Kinematics and Dynamics of Galaxies* (ed. Athanassoula, E.) 3–8 (IAU Symp. 100, Reidel, Dordrecht, 1982).
9. Steidel, C. C., Dickinson, M. & Persson, S. E. *Astrophys. J.* **437**, L75–L78 (1994).

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Stress-induced domain reorientation in urea inclusion compounds

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FOR over 50 years urea inclusion compounds (UICs)—in which molecules are entrapped within the cavities of a hydrogen-bonded urea network—have intrigued chemists, crystallographers and spectroscopists¹. Most previous work on these and other inclusion compounds has focused on intrachannel interactions and dynamics. We have now found that UICs can serve as useful models to probe long-range interactions and cooperative phenomena in crystals. Here we report the structure of 2,10-undecanedione/urea (1:9) (1/urea), in which an extended hydrogen-bonded array connecting hosts and guests serves to distort the urea channel away from the hexagonal symmetry normally observed for UIC crystals. The distortion is large enough, and exhibits enough cooperativity, to generate macroscopic domains, but is small enough to allow facile domain reorientation under small compressive stresses. By incorporating a specific impurity (2-undecanone) into 1/urea, we can

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modify the domain size and optical properties, and make the domain reorientation spontaneously reversible when the uniaxial stress is released. All of these phenomena can be understood in terms of cooperative interactions between different channels of the hydrogen-bonded network formed by urea and its guest.

In most UICs the urea molecules are connected by hydrogen bonds to form helical ribbons, which are woven together to form an array of linear, hexagonal tunnels that contain straight-chain hydrocarbons and analogues^{2,3}. With few exceptions^{4–6}, the host structure exists in either of two chiral space groups, $P6_322$ or $P6_522$. Because the channels are smooth and rigid, guest molecules are held loosely and undergo substantial torsions, librations^{7,8} and translations^{9,10}, as well as diffusive motions or jumps about the six-fold axis¹¹. Urea forms non-stoichiometric inclusion compounds with a wide variety of long-chain guests, which typically pack within van der Waals contact of each other to give an incommensurate spacing¹². That is, there are no reasonably small integers m and n that satisfy the relationship $mc_g = nc_h$, where c_h and c_g are the host and guest repeats along the channel axis¹³.

With most incommensurate UICs, static and dynamic disorder of the guest molecules maintains the average hexagonal symmetry of the host structure. In crystals of 1/urea, however, the host and guest are commensurate ($2c_g = 3c_h$), and the molecules are aligned in macroscopic domains of orthorhombic symmetry (space group $C222_1$). Within the channels, guest molecules lie with their mean planes roughly parallel to (010) and with their carbonyls nearly along [100]. The two-fold screw axis that relates the guest molecules arises as a specific consequence of the metric properties of the host–guest system. This lowered symmetry distorts the channel so that the interchannel separation along [100] is 8.345 Å, not 8.22 Å as in the undistorted hexagonal structure. A complementary 2% shrinkage occurs along [010]. Unlike the hexagonal crystals that are formed by almost all other UICs, crystals of 1/urea are birefringent when viewed along [001] between crossed polars. This birefringence reveals a rare 12-fold sectoring, in which adjacent domains are related by $\sim 60^\circ$ rotation of the guest and concomitant distortion of the host. This twinning occurs across two different families of boundaries ($\{130\}$ and $\{110\}$) to generate a total of 12 sectors (Fig. 1a).

A view close to the channel axis of 1/urea (Fig. 2b) presents a mechanism for formation of macroscopic domains: along the channel wall that forms (100), every third urea molecule turns about its two-fold axis so that its *syn* hydrogens make weak hydrogen bonds with guest carbonyls in adjacent channels. These ureas tether guests with different z coordinates (Fig. 2c) to make a hydrogen-bonded array that extends along [001]. This constitutes a continuously bonded chain that links two adjacent channels and requires them to distort in parallel. Crystals of 2-undecanone/urea (2/urea; see Fig. 2a) exhibit the same type of interchannel guest ordering as 1/urea¹⁴, but the absence of the

second carbonyl gives rise to orientational averaging of the guests and a lowering of interchannel cooperativity to give a normal hexagonal structure. The optically uniaxial crystals of 2/urea (not shown) stand in contrast with UICs grown from a 1:1 mixture of 1 and 2 (dissolved in CH_3OH), which often exhibit hundreds of birefringent sectors (Fig. 1b).

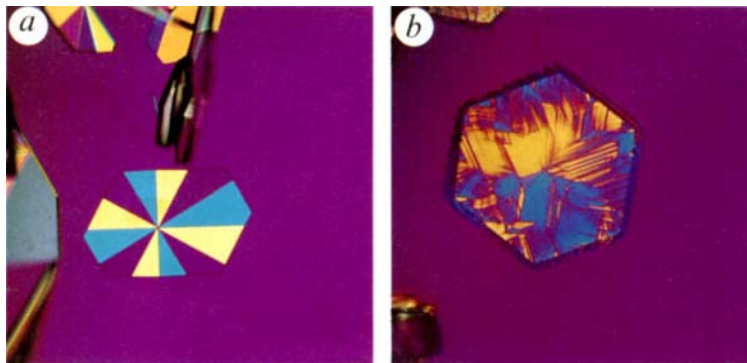
Crystals of 1/urea exhibit striking ferroelastic^{15,16} properties. If a small stress is applied to a (110) face of 1/urea, large domains are generated in which the optical indicatrix has been reoriented in the (001) plane, typically by multiples of $\sim 60^\circ$ (Fig. 3). Upon application and subsequent release of very light stresses, transient (daughter) phases grow and then retreat in a matter of seconds as they are engulfed by the original (mother) phase. With somewhat greater pressure, daughter phases are formed irreversibly, but the mother phase can often be regenerated by simply rotating the crystal by $\sim 60^\circ$ and applying stress to a (110) face of the daughter.

These observations are readily understood in terms of reorientation of guest molecules about the channel axis to give a new orthorhombic phase in which guests lie with their carbonyls perpendicular to the applied stress. During this process, each guest molecule must break two hydrogen bonds to the host, then rotate by $\sim 60^\circ$ to form two new hydrogen bonds with urea molecules on an adjacent channel wall. At the same time, the two urea molecules making contact with the guest must rotate back towards the (100) plane that forms the channel wall, and two others on an adjacent wall must turn to make hydrogen bonds with the newly oriented guest. Because the urea molecules making contact with the guests are tethered on both sides of a channel wall, this molecular reorientation is translated in a cooperative way into a full-scale phase reorientation.

By incorporating 2-undecanone impurities into 1/urea, we can tailor the cooperativity and dynamics of this stress-induced phase reorientation. Thus far, upon application and release of stress (in some instances almost to the breaking point of the crystal), mixed UICs of 1 (80%) and 2 (20%) have always shown rapid return to the mother phase from the daughter phase (Fig. 4). To understand this dramatic change from plastic to elastic deformation on introduction of the 2-undecanone impurity, one must consider the stabilities of the mother and daughter phases as well as the interface that connects them. Because the impurities break up the host–guest hydrogen bonding, and the cell parameters shift slightly towards hexagonal, we conclude that the cohesive forces that hold the daughter phase together (and thus resist regeneration of the mother phase) are lower than in 1/urea. By the same token, however, the unit strain imposed by the mother phase is correspondingly lower, so one may deduce that the passage back to the mother phase occurs through a lower barrier than in 1/urea.

Because the phase reorientation is a heterogeneous phenomenon that occurs at the interface between the two phases, the barrier to reorientation varies according to the structure, compo-

FIG. 1 a, Photomicrograph of 1/urea along [001] showing 12-fold sectoring (under crossed polars and $\lambda/4$ plate). Convolution of the six-fold rotation between sectors and the two-fold screw axis within a sector gives rise to three distinguishable orientations of the optical indicatrix and a maximum of three interference colours for a given crystal thickness. At the outer edge of the crystal, alternate sector boundaries protrude ($\{130\}$ interfaces (vertical boundary at the top)) or intrude ($\{110\}$ interfaces). These reflect the extensions or contractions of the unit-cell boundaries in this distorted cell. b, Photomicrograph of mixed UIC of 1 and 2 ($\sim 1:1$) taken under the same conditions. (Magnification in a and b, $\times 68.6$.)



sition and history of the interfacial environment. Together, these determine the cooperativity and dynamics of the process. At a local level, regeneration of the mother phase requires it to intrude into the daughter phase and overcome the cooperative resistance that keeps the latter phase intact. This intruding mother phase can sustain itself within the daughter (and pass through the barrier for phase reorientation) only if it reaches a critical size and shape. The 2-undecanone impurities act to lower the barrier in two related ways: by providing 'nucleation' sites where rotation of the guest is facilitated by the absence of a hydrogen-bond acceptor, and by severing the links within the cooperative networks that must be reoriented under the strain imposed by the mother phase. In essence, removal of 10% of the hydrogen bonds in the impure crystals serves to reduce the criti-

cal size that the intruding domain must reach before it can co-exist at the interface with the retreating daughter phase.

Most discussions of impurity hardening revolve around a stressed defect model in which the impurity (for example, carbon in steel) makes the system more elastic by imposing a local stress that counters the applied stress¹⁷. In contrast, the present example shows how impurities can effectively 'catalyse' an elastic response in an organic crystal by providing nucleation sites for the reorientation and by disrupting the cooperative networks that are broken and formed during deformation. Combined with the versatility of organic synthesis, a knowledge of the commensurate relationships within this series of inclusion compounds should allow us to engineer ferroelastic crystals containing different types of host-guest networks. If the same crystal

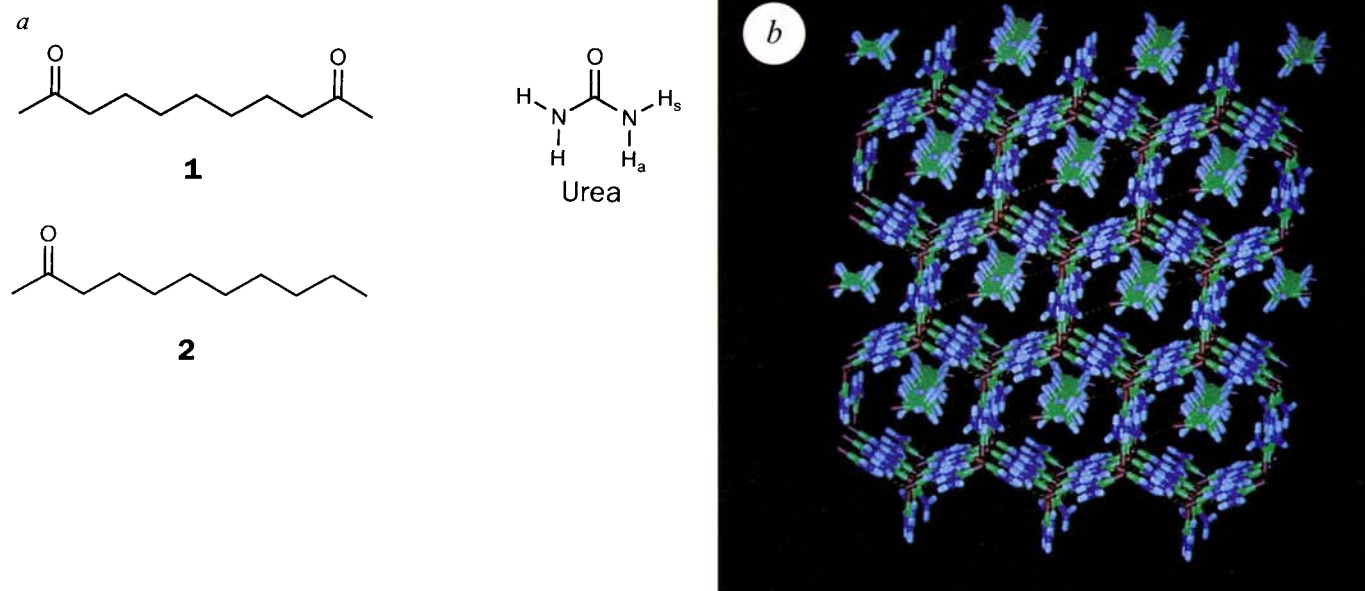
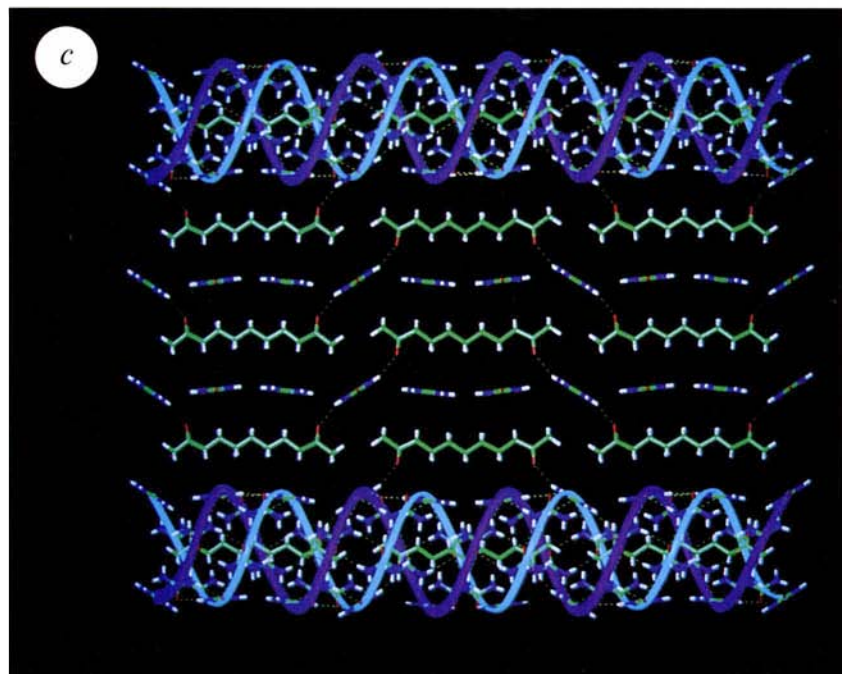


FIG. 2 *a*, Molecular structure of urea and guests **1** and **2**. *b*, A view down the channel of **1**/urea ([010] vertical). Along the vertical walls, every third urea turns into the channel to make weak hydrogen bonds with the guest. This can be seen more clearly in a cut-away view of the (010) plane (*c*), which shows the channel structure and host-guest hydrogen bonding. Within a channel, guests are related by a 2_1 axis. Urea molecules tether guests in adjacent channels by diagonal hydrogen bonds. Distances (Å) and angles for these hydrogen bonds are as follows (H_s , *syn* hydrogen of urea): O $\cdots$ H $_s$ distance, 2.351(6); N-H $_s$ distance, 0.90; C-O distance, 1.184(8); O $\cdots$ N distance, 3.018(6) Å; O-H $_s$ -N angle, 131.3(6)°; C-O-H $_s$ angle, 147(1)°. (Cell constants (Å) are $a=8.345(1)$, $b=13.939(3)$, $c=32.982(6)$.) Crystallographic data for **1**/urea are available: see Supplementary Information.



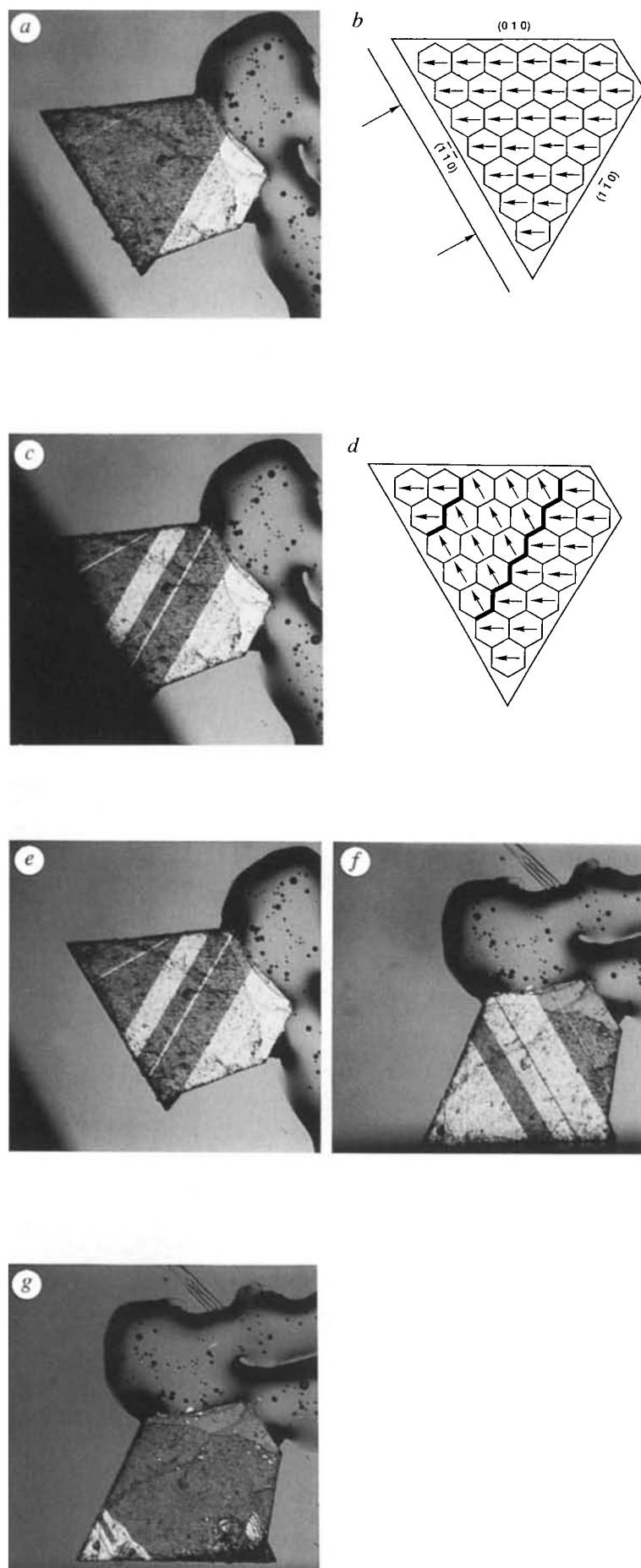
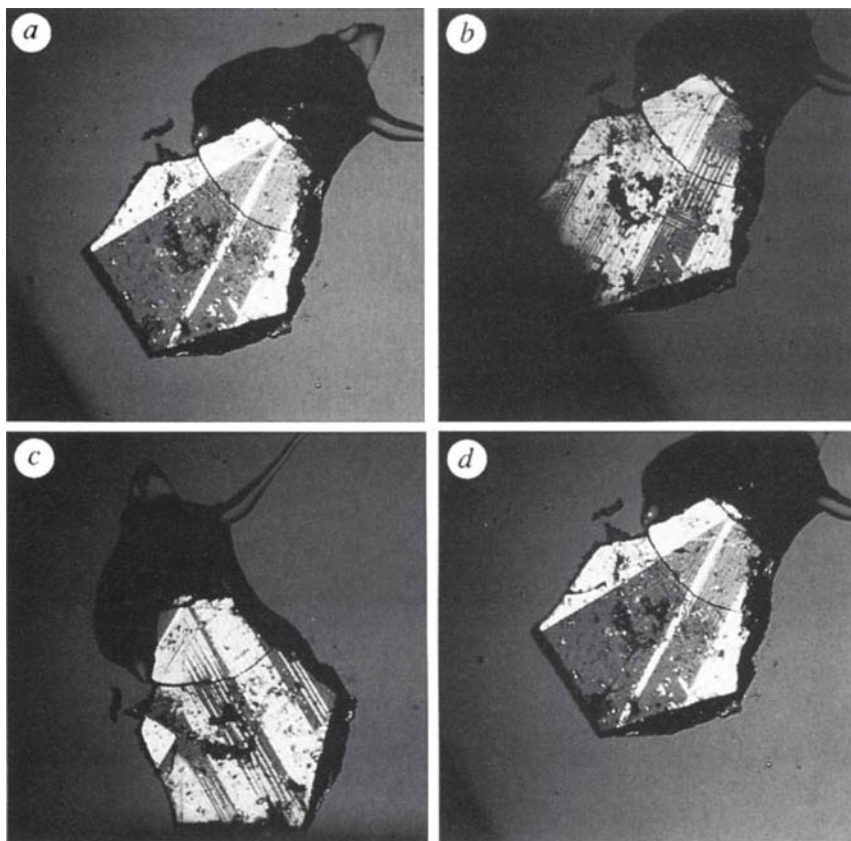


FIG. 3 Irreversible stress-induced phase reorientation in **1**/urea (under crossed polars and $\lambda/4$ plate). *a*, Crystal fragment oriented as shown in *b*, before stress. The upper right-hand corner has been fastened with epoxy resin. *b*, Schematic depiction of **1**/urea before stress. Arrows represent carbonyl dipoles for guests in one layer. *c*, **1**/urea under fairly uniform stress along the $(\bar{1}10)$ face. *d*, Schematic depiction of domain reorientation. *e*, As in *c*, after removal of stress. *f*, As in *c*, but rotated by 60° to show birefringence of new phase (dark strip). *g*, The same crystal after several applications of stress used to reorient $>80\%$ of the crystal to the daughter phase (dark in this orientation). Some damage was generated in the lower right-hand corner. (Magnification in *a*, *c*, *e*–*g*, $\times 17.1$.)

FIG. 4 Reversible stress-induced phase reorientation of UIC containing 80% **1** and 20% **2**. The crystal orientation is the same as in Fig. 3. *a*, Before stress. *b*, Under stress as in Fig. 3c. *c*, As in *b*, but rotated by 60° to show birefringence of new domains. Note the high dislocation density¹⁸ along the boundaries between mother and daughter phases. *d*, After stress. The irreversible retreat to the mother phase is typically complete within one second. (Magnification in *a*–*d*, $\times 19.3$.)



engineering techniques can also be used to generate ferroelectric inclusion compounds, it should be possible to exploit the use of functional-group substitutions and impurities to gain a better understanding of the cooperative forces involved in ferroelectric switching processes. □

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1. Takemoto, K. & Sonoda, N. in *Inclusion Compounds* Vol. II (eds Atwood, J. L., Davies, J. E. D. & MacNicol, D. D.) 47–67 (Academic, London, 1984).
2. Smith, A. E. *Acta crystallogr.* **5**, 224–235 (1952).
3. Harris, K. D. M. & Thomas, J. M. *J. chem. Soc., Faraday Trans.* **86**, 2985–2996 (1990).
4. Hollingsworth, M. D., Santarsiero, B. D. & Harris, K. D. M. *Angew. Chem. int. Edn engl.* **33**, 649–652 (1994).
5. Hollingsworth, M. D., Brown, M. E., Santarsiero, B. D., Huffman, J. C. & Goss, C. R. *Chem. Mater.* **6**, 1227–1244 (1994).
6. Otto, J. *Acta crystallogr.* **B28**, 543–551 (1972).
7. Greenfield, M. S., Vold, R. L. & Vold, R. R. *J. chem. Phys.* **83**, 1440–1443 (1985).
8. Greenfield, M. S., Vold, R. L. & Vold, R. R. *Molec. Cryst. liq. Cryst.* **66**, 269–298 (1989).
9. Guillaume, F., Smart, S. P., Harris, K. D. M. & Dianoux, A. J. *J. Phys.: Condens. Matter* **6**, 2169–2184 (1994).
10. Schmicker, D., van Smaalen, S., de Boer, J. L., Haas, C. & Harris, K. D. M. *Phys. Rev. Lett* **74**, 734–737 (1995).
11. Harris, K. D. M. *J. Solid St. Chem.* **106**, 83–98 (1993).
12. Lenné, H. U., Mez, H. C. & Schlenk, W. *J. Justus Liebigs Annin Chem.* **732**, 70–96 (1970).
13. Rennie, A. J. O. & Harris, K. D. M. *J. chem. Phys.* **96**, 7117–7124 (1992).
14. Hollingsworth, M. D. & Goss, C. R. *Molec. Cryst. liq. Cryst.* **219**, 43–62 (1992).
15. Abrahams, S. C. *Mater. Res. Bull.* **6**, 881–890 (1971).
16. Salje, E. K. H. *Phase Transitions in Ferroelastic and Co-elastic Crystals* (Cambridge Univ. Press, 1990).
17. Callister, W. D. Jr *Materials Science and Engineering: an introduction* (Wiley, New York, 1994).
18. Bornarel, J., Lajzerowicz, J. & Legrand, J. F. *Ferroelectrics* **7**, 313–314 (1974).

SUPPLEMENTARY INFORMATION. Requests should be addressed to Mary Sheehan at the London editorial office of Nature.

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Potential accumulation of a CFC-replacement degradation product in seasonal wetlands

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BECAUSE of their refractory nature, chlorofluorocarbons (CFCs) released by industries are eventually transported to the stratosphere, where they are slowly degraded by solar ultraviolet radiation into highly reactive chlorine atoms which can then participate in a catalytic ozone depletion cycle. For this reason, signatories to the Montreal Protocol and subsequent amendments have agreed to phase out the use of CFCs¹ in the next few decades. Hydrofluorocarbons and hydrochlorofluorocarbons have been proposed as CFC replacements; atmospheric degradation of several of these is expected to produce trifluoroacetate (TFA), which is removed from the atmosphere mainly by rain^{2,3}. The global average TFA concentration in rain water for the year 2010 is estimated⁴ to be $0.16 \mu\text{g l}^{-1}$ —well below the concentrations thought to inhibit plant growth ($\sim 10^2$ – $10^6 \mu\text{g l}^{-1}$)⁵. But our modelling analysis, presented here, indicates that in conditions of high evapotranspiration, TFA could attain appreciable concentrations ($>10^2 \mu\text{g l}^{-1}$) in the local surface waters of seasonal wetlands within a few decades, if removal by degradation and seepage is limited.

Since 1988, research has focused on tropospheric degradation pathways of the newer CFC replacements, most of which are halogenated ethane derivatives. Although laboratory studies indicated that a number of these replacements would degrade to C_1 products, three were found⁶ to yield trifluoroacetyl halides of the form $CF_3C(O)X$, where X is Cl or F. These are HCFC-123 (CCl_2HCF_3), HCFC-124 ($CClH_2CF_3$) and HFC-134a (CF_3CFH_2) (Fig. 1).

Hydrolysis of these acetyl halides in cloud water is expected to form trifluoroacetic acid ($CF_3C(O)OH$) which is subsequently removed mainly by wet and dry deposition. Trifluoroacetate seems to be stable in water and no significant loss process such as hydrolysis, photolysis or formation of insoluble salts has been identified^{7,8}. Therefore, TFA could accumulate in local surface waters in the absence of major biological or physical removal processes.

Questions were first raised in 1989 about the lack of information on the effect TFA might have on the environment⁶. Subsequent research^{9,10} has found that TFA, added as the sodium salt, inhibits growth in one or two species of alga at low concentra-

tions ($100\text{--}300\text{ }\mu\text{g l}^{-1}$) out of the seven tested species. The other species showed no growth inhibition below $1\text{--}2\text{ g l}^{-1}$ TFA. TFA has been observed to bioconcentrate in plant tissues⁵ and to inhibit early plant growth in sunflowers, mung beans and wheat at concentrations down to 1 mg kg^{-1} dry soil¹¹. Assuming the soil is 50% solid, 50% moisture by weight, the equivalent TFA water concentration would be $1,000\text{ }\mu\text{g l}^{-1}$.

The projected global average TFA rainwater concentration^{2,4} suggests that TFA concentration will be exceedingly low in the oceans. Also, the residence times will be too short for any substantive accumulation in freshwater ponds, lakes and streams. However, there are numerous water bodies^{12–14} characterized by little to no outflow and high evaporation rates (for example, agricultural evaporation ponds and seasonal wetlands including playa lakes, tundra ponds and vernal pools). This type of water body may have the potential to accumulate air- and rain-borne acids, including TFA. If these acids are not removed physically or chemically, their concentrations might be elevated by several orders of magnitude above the global average concentration in rain water. The ecological importance of many of these ubiquitous wetlands lies not only in their acting as habitat for many rare and endangered plant and animal species^{15,16}, but also in their use by migratory and wintering waterfowl for foraging and resting during winter and early spring¹⁷. Here, we identify the parameters that relate the rainwater concentration of salts and acids, exemplified by TFA, to the potential long-term accumulated concentration for a particular water body.

We can approximate the TFA concentration in the water body, N ($\mu\text{g l}^{-1}$), in the seasonal wetland assuming a first-order loss term to represent seepage, aeolian transport and/or biodegradation. The equation for the annual average value of N can be written in the form¹⁸,

$$dN/dt = -SN + P \quad (1)$$

where S (yr^{-1}) is the effective seepage and/or biodegradation rate (loss frequency), P is the annually averaged local source strength of TFA ($\mu\text{g l}^{-1}\text{ yr}^{-1}$) as defined in Fig. 2 legend and t is time. The loss term, S , may incorporate biodegradation, water flow out of the closed basin and the loss of material due to aeolian transport. A linear biodegradation rate is a common simplifying assumption made in many sedimentary biogeochemical models^{19,20}. If both P and S are constant in time, TFA will accumulate to P/S at steady state. Figure 2a shows the predicted TFA concentration in a water body after 50 years, as a function of S and P . We explore a wide range of seepage rates because of the limited information available concerning loss rates for various types of basins. Seepage rates have been assumed to be minimal in playa lakes^{14,21}, but are probably more significant in vernal pools²². In Fig. 2a, it is assumed that all TFA from the current season and previous seasons is available to biodegrade and seep. The values in Fig. 2b represent an upper limit, assuming no biodegradation and that all TFA from previous seasons is prevented from seeping, that is, labile TFA is trapped in the sediments and biota. Thus, only the incoming TFA for each year is available to seep. This assumption makes little difference if the seepage rates are less than 1% per year, but enables significantly larger accumulation for seepage rates greater than 1% per year.

One set of experiments suggests that TFA biodegradation might be concentration dependent²³. To model this data, we assume a cutoff concentration for biodegradation and a non-linear relation for the degradation rate. We may approximate the behaviour of N by the Riccati equation,

$$\begin{aligned} dN/dt &= f(N)N + P \\ f(N) &= -r(1 - N/K) \quad \text{for } N \leq K \\ f(N) &= 0 \quad \text{for } N > K \end{aligned} \quad (2)$$

where $f(N)$ is the biodegradation rate (in yr^{-1}), N is the concentration of TFA in the water body (in $\mu\text{g l}^{-1}$), t is time (in yr),

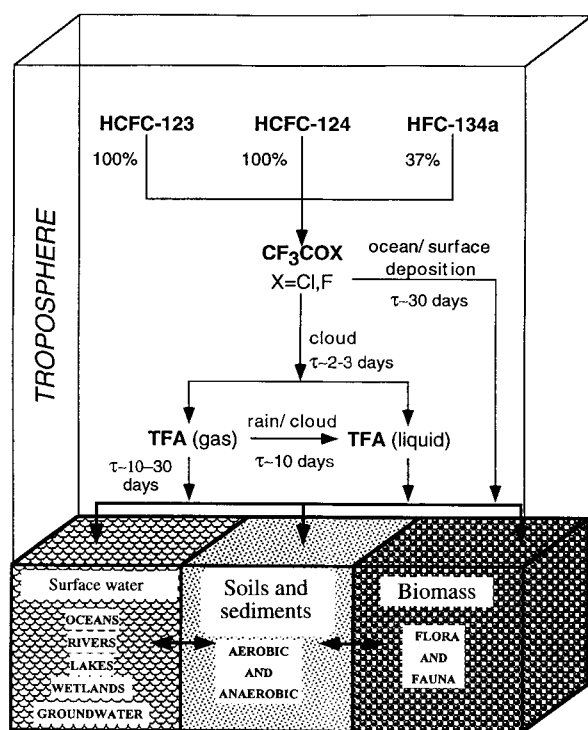


FIG. 1 Schematic degradation pathways for HCFC-123, HCFC-124 and HFC-134a. The global average time constant for each process is indicated by τ , and the yield of CF_3COX from the degradation of the precursors is also given²⁴. In the case of HFC-134a, 63% of the CF_3CHFO undergoes carbon-carbon bond cleavage before reacting with O_2 to form $CF_3C(O)F$ (ref. 31). The rate-limiting step for the production of $CF_3C(O)F$ is given by the reaction of OH with the precursor gas. For HCFC-123, τ is 1.4 yr; for HCFC-124, τ is 5.7 yr and for HFC-134a τ is ~ 17 yr. Most of the $CF_3C(O)F$ is hydrolysed in cloud water to form TFA which is released into the atmosphere when cloud water evaporates. At most, 5% of TFA may degrade² by reaction with OH radicals³. Wet deposition accounts for at least 75% of the total deposition². A global average TFA rainwater concentration of $0.16\text{ }\mu\text{g l}^{-1}$ was estimated⁴ for the year 2010 by adopting the emission scenario for precursor emissions provided by EPA (D. Hufford, personal communication), corresponding to global emissions of 23.3 kt of HCFC-123, 49.2 kt of HCFC-124 and 221.5 kt of HFC-134a in the year 2010, TFA yields of 0.37, 1.0 and 1.0 by mass estimated for HFC 134a, HCFC-124 and HCFC 123 (V. R. Kotamarthi, personal communication) and a global rainfall rate³² of $5 \times 10^{17}\text{ l yr}^{-1}$.

r is the biodegradation rate (loss frequency, in yr^{-1}) and K ($\mu\text{g l}^{-1}$) is the TFA concentration at which biodegradation ceases.

The general solution to equation (2) may be viewed as various surfaces in parameter space. The solution of equation (2), for arbitrary initial values of N_0 is described in Box 1. Our analysis for the cases assuming no initial TFA concentration, that is, $N_0 = 0$, reveals three possible states characterizing three distinct modes of accumulation for TFA that depend on the sign of the critical parameter $\delta = r(4P/K - r)$, where δ is the discriminant of the quadratic equation $f(N)N + P = 0$. If $\delta \leq 0$ (or $P/K \leq r/4$), then N will increase monotonically with time but is bounded by $K/2$. If $\delta > 0$ (or $P/K > r/4$), then TFA will reach the value K in a finite amount of time, after which N will continue to increase linearly with time.

Figure 3 shows the results in the phase space (P - r domain) for the case $N_0 = 0$ and $K = 100 \mu\text{g l}^{-1}$ as suggested by the results of Visscher *et al.*²³. The line $\delta = 0$ separates the 'population' into an accumulation state (that is, $\delta > 0$) and a non-accumulation state (that is, $\delta < 0$). The time required for TFA to accumulate to $100 \mu\text{g l}^{-1}$ is also plotted in Fig. 3 as a function of P and r . For $P = 5 \mu\text{g l}^{-1} \text{yr}^{-1}$ ($R_{\text{TFA}} = 1 \mu\text{g l}^{-1}$ and $E = 5 \text{ yr}^{-1}$) and $r = 0.1 \text{ yr}^{-1}$, N reaches $100 \mu\text{g l}^{-1}$ in about 30 years. On the other hand, for $P < 2.5 \mu\text{g l}^{-1} \text{yr}^{-1}$ and $r > 0.1 \text{ yr}^{-1}$, no accumulation is expected.

Although assessment of TFA accumulation in a seasonal wetland is a complex issue, our model suggests that for a first-order description, the parametric space may be represented by: (1) the local concentration of TFA in rainfall, R_{TFA} , which depends on local meteorological conditions and local fluxes of precursors; (2) the evaporative concentration factor, E , occurring in the seasonal wetland; (3) the seepage rate, S and (4) the biodegradation rate, $f(N)$, which is dependent on the degradation rate r and the concentration at which biodegradation ceases, K . These representations will now be considered in more detail.

(1) Recent three-dimensional model results predict annual average TFA concentrations in rain water in arid and semi-arid regions to be a factor of 2-4 times larger than the global average²⁴. There are many conditions that could combine to enhance R_{TFA} above the background average values. Earlier measurements of CFCs in urban areas were found to be factors of 10-20 greater than the zonal average²⁵⁻²⁷; we would assume that the HCFC/HFC compounds could be higher by a similar amount. In addition, in urban areas the atmospheric $\text{OH}^{\bullet}$ levels could sometimes be as much as a factor of 10 larger than the zonal mean concentration^{28,29}. This means that locally the production rate of the TFA precursors (a quantity proportional to the product of local $\text{OH}^{\bullet}$ and precursor concentrations) could be enhanced up to two orders of magnitude above the zonal average.

(2) Local evapotranspiration further enhances local concentration. Data on the concentration of solutes, for example, Cl, K, Na, Mg and Ca, can be used to constrain the evaporation rate. For wetlands that exhibit a solute enhancement, the value for E could be as little as a factor of 2 to greater than 40 depending upon the type of seasonal wetland¹⁸. Future studies should include a more detailed assessment of E .

(3) Assessing the fraction of TFA loss due to seepage from a water-body system is crucial. Because TFA has been observed to bioaccumulate^{5,30} and adsorb onto certain soil types (C. Driscoll, personal communication), it will be important to determine the amount of TFA actually lost to seepage compared to the amount stored in plant tissues or soils, and whether the TFA is labile or non-labile.

(4) Although current research indicates that the significance of biodegradation in limiting potential build-up of TFA is still highly uncertain (M. Odom and R. Oremland, personal communication), it is still necessary to ascertain the products of such degradation processes, the rate at which TFA degrades as a function of concentration under natural conditions for a given degradation regime (for example, oxic, sulphate-reducing and

BOX 1 Analytical solutions to equations (1) and (2)

The solution to equation (1) is given by:

$$N(t) = \frac{P}{S} (1 - e^{-St}) + N_0 e^{-St} \text{ where } N(0) = N_0$$

The steady-state solution for $N(t)$ is P/S .

The solution to equation (2) depends on the value of N_0 .

$$\text{For } N_0 \geq K, N(t) = N_0 + Pt$$

For $N_0 < K$, the solutions depend on the values of δ , r and N_0 . Three cases may be identified.

$$\text{Case 1. } \delta < 0, r > \frac{4P}{K}, \frac{\sqrt{-\delta}}{r} < 1$$

$$N(t) = \frac{K}{2} \left(1 + \frac{\sqrt{-\delta}(A + B e^{\sqrt{-\delta}t})}{r(A - B e^{\sqrt{-\delta}t})} \right)$$

$$A = N_0 - \frac{K}{2} (1 - \sqrt{-\delta}/r) \quad B = N_0 - \frac{K}{2} (1 + \sqrt{-\delta}/r)$$

$$(a) \text{ For } N_0 < \frac{K}{2} (1 - \sqrt{-\delta}/r)$$

$$\lim_{t \rightarrow \infty} N(t) = \frac{K}{2} (1 - \sqrt{-\delta}/r)$$

$$(b) \text{ For } N_0 = \frac{K}{2} (1 - \sqrt{-\delta}/r)$$

$$N(t) = \frac{K}{2} (1 - \sqrt{-\delta}/r), \text{ 'steady solution'}$$

$$(c) \text{ For } \frac{K}{2} (1 - \sqrt{-\delta}/r) < N_0 < \frac{K}{2} (1 + \sqrt{-\delta}/r)$$

$$\lim_{t \rightarrow \infty} N(t) = \frac{K}{2} (1 - \sqrt{-\delta}/r)$$

$$(d) \text{ For } N_0 = \frac{K}{2} (1 + \sqrt{-\delta}/r)$$

$$N(t) = \frac{K}{2} (1 + \sqrt{-\delta}/r), \text{ 'steady solution'}$$

$$(e) \text{ For } N_0 > \frac{K}{2} (1 + \sqrt{-\delta}/r)$$

$$N(t) \rightarrow K \text{ at time } t_K = \frac{1}{\sqrt{-\delta}} \ln \frac{4(1 - \sqrt{-\delta}/r)}{B(1 + \sqrt{-\delta}/r)}$$

$$\text{For } t > t_K, N(t) = P(t - t_K) + K$$

$$\text{Case 2. } \delta = 0, \frac{P}{K} = \frac{r}{4}$$

$$N(t) = \frac{K}{2} - \frac{(2N_0 - K)K}{(2N_0 - K)t - 2K}$$

$$(a) \text{ For } N_0 < K/2$$

$$\lim_{t \rightarrow \infty} N(t) = K/2, \text{ goes } \approx \text{ as } 1/t$$

$$(b) \text{ For } N_0 = K/2, N(t) = K/2, \text{ for } \forall t$$

$$(c) \text{ For } K < N_0 < \frac{K}{2}$$

$$N(t) \rightarrow K \text{ at time } t_K = \frac{4N_0}{(2N_0 - K)r}$$

$$\text{For } t > t_K, N(t) = P(t - t_K) + K$$

$$\text{Case 3. } \delta > 0, \frac{4P}{K} > r$$

$$N(t) = \frac{K}{2} \left[1 + \frac{\sqrt{\delta}}{r} \left(\frac{\tan \sqrt{\delta}t/2 + \frac{2r}{K\sqrt{\delta}} (N_0 - K/2)}{1 - \frac{2r}{K\sqrt{\delta}} (N_0 - K/2) \tan \sqrt{\delta}t/2} \right) \right]$$

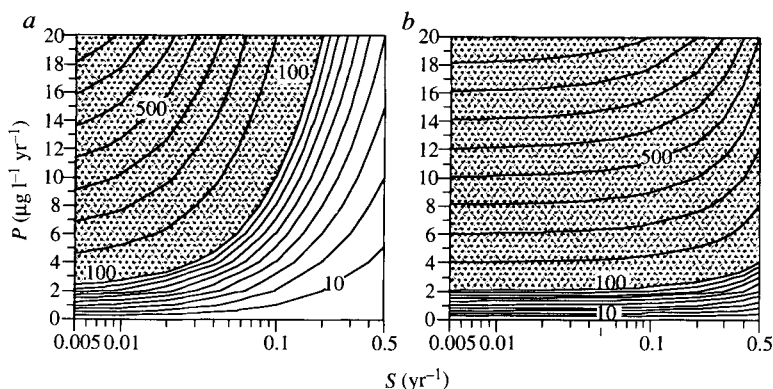
$$\text{valid for } \forall N_0 \leq K, N(t) \leq K$$

$$N(t) \rightarrow K \text{ at time } t_K$$

$$= \frac{2}{\sqrt{\delta}} \left(\tan^{-1} \frac{r(200 - K)}{K\sqrt{\delta}} - \tan^{-1} \left(\frac{2rN_0}{K\sqrt{\delta}} - \frac{r}{\sqrt{\delta}} \right) \right)$$

$$\text{For } t > t_K, N(t) = P(t - t_K) + K$$

FIG. 2 TFA concentration (contours; $\mu\text{g l}^{-1}$) in the soil pore space after 50 years of accumulation as a function of the loss rate, S (yr^{-1}), and the annually averaged local source strength of TFA, P ($\mu\text{g l}^{-1} \text{yr}^{-1}$). The TFA concentration in the water body, N ($\mu\text{g l}^{-1}$), is controlled by the amount of TFA put into the pool from rain in the wet season and the evapotranspiration rate (the loss of water through evaporation and plant respiration) that determines the amount of water left in the pool sediments during the dry season. We define V_{rain} ($\text{m}^3 \text{yr}^{-1}$) as the volume of rain water entering the pool over one year, and V_{pool} (m^3) as the volume of water remaining in the underlying soil pore space at the end of the wet season. The amount of TFA added in one year is $R_{\text{TFA}} V_{\text{rain}} \times (1 \text{ year})$ where R_{TFA} ($\mu\text{g l}^{-1}$) is the concentration of TFA in rain. After evaporation, N will increase by $(R_{\text{TFA}} V_{\text{rain}} \times (1 \text{ year}) / V_{\text{pool}})$. The annual rate of increase for N is given by $R_{\text{TFA}} E$, where E (yr^{-1}) is the annual evapotranspiration rate defined as $E = V_{\text{rain}} / V_{\text{pool}}$. E is a function of, for example, surface area, depth, geometry, inflowing channel conditions of the individual wetland, and climate factors such as the daily precipitation rate, relative humidity and cloudiness. *a*, During the wet season, all TFA stored in soil pore space from previous seasons is assumed to be instantly released into overlying water and available to seep out of the water body. *b*, The effective concentration of TFA ($\mu\text{g l}^{-1}$) in the soil pore space after



50 yr of accumulation. During the wet season, all previous season's TFA is assumed to be bound to soils or stored in plants and thereby unavailable to seep. Only TFA from the present season seeps out of the system. The shaded regions in both *a* and *b* indicate combinations of P and S that may result in TFA concentrations that affect plant growth.

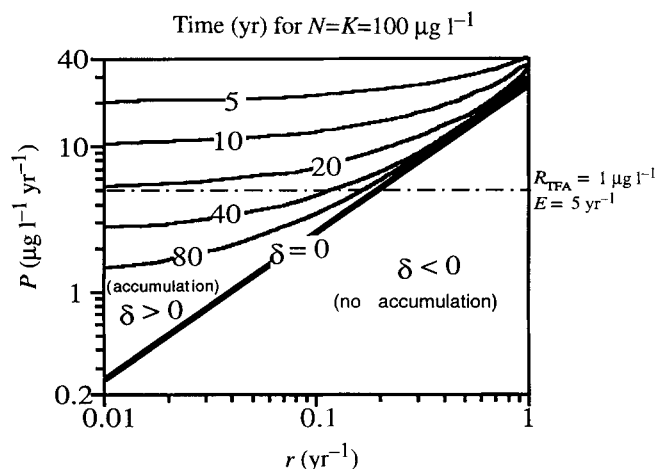


FIG. 3 The increase in TFA concentration in the wetland is determined by values of P , S , r and K that characterize the system. Here we assume that no seepage occurs. The figure illustrates how the combination of values for P and r determines the value of δ for $K=100$ ($\mu\text{g l}^{-1}$). The line $\delta=0$ defines the pair of values (P , r) on the line $P=Kr/4$. The solution given in Box 1 shows that given $N_0=0$, N approaches $K/2$ for $\delta=0$ and $K/2(1-\sqrt{-\delta/r})$ for $\delta<0$. For $\delta>0$, the contours plotted show the time needed for TFA to reach K ($100 \mu\text{g l}^{-1}$ in this example) as a function of P and r . After K is reached, TFA will continue to accumulate unabated because no other degradation mechanism is assumed. The TFA concentration will continue to increase at the rate of $P \mu\text{g l}^{-1} \text{yr}^{-1}$.

methanogenic), and the likelihood of encountering each regime in seasonal wetlands.

Although we believe that TFA would not have any biological effects in seasonal wetlands if its concentration is limited to the projected global average rainwater concentration, our model indicates that TFA could be concentrated locally well above those values. Consequently, it is prudent to gather additional data to ascertain the accumulation rate of TFA in wetlands and its biological effects. □

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1. Parson, E. A. & Greene, O. *Environment* **37**, 16–43 (1995).
2. Kanakidou, M., Dentener, F. J. & Crutzen, P. J. *J. geophys. Res.* (in the press).
3. Mogelberg, T. E., Nielsen, O. J., Sehested, J., Wallington, T. J. & Hurley, M. D. *Chem. Phys. Lett.* **226**, 171–177 (1994).
4. Kotamarthi, V. R. et al. (abstr.) *Eos* **75**, 137 (1994).
5. Thompson, R., Stewart, K. M. & Gillings, E. Report SP91-18.8 (Alternative Fluorocarbons Environmental Acceptability Study (AFEAS), Washington DC, 1994).
6. *Scientific Assessment of Stratospheric Ozone: 1989 Global Ozone Research and Monitoring Project*, Vol. II (Rep. No. 20, World Meteorological Organization, Geneva, 1989).
7. *Proc. AFEAS Workshop on Decomposition of TFA in the Environment* (ed. Magid, H.) (AFEAS, Washington DC, 1994).
8. *Proc. Workshop on the Environmental Fate of Trifluoroacetic Acid* (ed. Chumley, F. G.) (AFEAS, Miami Beach, 1994).
9. Groeneweld, A. H. C., de Kok, H. A. M. & van den Berg, G. Report CTR91-18.9 (AFEAS, Washington DC, 1993).
10. Groeneweld, A. H. C. & Berends, A. G. Report CTR91-18.20 (AFEAS, Washington DC, 1995).
11. Thompson, R. S. *AFEAS Workshop on the Environmental Fate of Trifluoroacetic Acid* (ed. Chumley, F. G.) Ch. 15 (AFEAS, Miami Beach, 1994).

12. Snyder, C. T. *Int. Ass. Sci. Hydrol.* **7**, 53–59 (1962).
13. Hardie, L. A. & Eugster, H. P. *Spec. Pap. Miner. Soc. Am.* **3**, 273–290 (1970).
14. Rosen, M. R. *Spec. Pap. geol. Soc. Am.* **289**, 1–18 (1994).
15. Robie, S. M. *Massachusetts Wildlife* **1**, 23–28 (1989).
16. Shortelle, A. B., Dudley, J. L., Prynoski, B. & Boyajian, M. *Am. Wat. Resour. Ass.* **28**, 463–471 (1989).
17. Sefchick, J., Skorupa, J. & Schwarzbach, S. Rep. CTR91-18.6 (AFEAS, Washington DC, 1994).
18. Haan, C. T., Johnson, H. P. & Brakensiek, D. L. (eds) *Hydrologic Modeling of Small Watersheds* 3–293 (Monogr. no. 5, Am. Soc. Agricultural Engineers, St Joseph, MI, 1982).
19. Berner, R. A. *Early Diagenesis: A Theoretical Approach* (Princeton Univ. Press, 1980).
20. Tromp, T. K., Van Cappellen, P. & Key, R. M. *Geochim. cosmochim. Acta* **59**, 1259–1283 (1995).
21. Eugster, H. P. & Jones, B. F. *Am. J. Sci.* **279**, 609–631 (1979).
22. Zedler, P. H. *Biological Rep.* 85, U.S. Fish and Wildlife Service (US Dept Interior, Washington DC, 1987).
23. Visscher, P. T., Culbertson, C. W. & Oremland, R. S. *Nature* **369**, 729–731 (1994).
24. Kotamarthi, V. R. et al. *J. geophys. Res.* (submitted).
25. Simmonds, P. G., Kerrin, S. L., Lovelock, J. E. & Shair, F. H. *Atmos. Envir.* **8**, 209–216 (1974).
26. Singh, H. B., Salas, L., Shigeishi, H. & Crawford, A. *Atmos. Envir.* **11**, 819–828 (1977).
27. Bastable, H. G., Rogers, D. P. & Schorran, D. E. *Atmos. Envir.* **24B**, 137–151 (1990).
28. Sillman, S., Logan, J. A. & Wofsy, S. C. *J. geophys. Res.* **94**, 1837–1851 (1990).
29. Satsumabayashi, H., Kurita, H., Chang, Y.-S., Carmichael, G. R. & Ueda, H. *Atmos. Envir.* **26A**, 2835–2844 (1992).
30. Frank, H. *AFEAS Workshop on the Environmental Fate of Trifluoroacetic Acid* (ed. Chumley, F. G.) Ch. 4 (AFEAS, Miami Beach, 1994).
31. Franklin, J. *Chemosphere* **27**, 1565–1601 (1993).
32. Palmer, E. & Newton, C. W. *Atmospheric Circulation Systems* (Academic, New York, 1969).

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Induction of epidermis and inhibition of neural fate by Bmp-4

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DURING gastrulation in vertebrates, ectodermal cells choose between two fates, neural and epidermal. The nervous system forms in response to signals from the Spemann organizer^{1,2}; ectoderm that does not receive these signals becomes epidermis. Unexpectedly, however, in *Xenopus*, neural tissue also forms when cell-cell communication within the ectoderm is disrupted by cell dissociation^{3,4} or by antagonists of the growth factor activin⁵⁻⁷. These observations suggest that epidermal specification depends on local signalling, by activin or a close relative, and that neural tissue forms when this communication is blocked⁶. Here we report that bone morphogenesis protein 4 (Bmp-4), a relative of activin that is expressed in the embryo at the time of ectodermal fate determination^{8,9}, is a potent epidermal inducer and neural inhibitor, the first reported in any vertebrate. Activin can inhibit neuralization by inducing mesoderm, but does not induce epidermis. Moreover, the dominant-negative activin receptor, which stimulates neuralization when expressed in the embryo^{5,6}, blocks Bmp-4 in our assay. Our findings demonstrate that epidermal fate can be induced, and thus provide further evidence that neural specification is under inhibitory control in vertebrates.

Xenopus ectoderm is neuralized by prolonged dispersal^{3,4}. If this change in fate results from the loss of a secreted epidermalizing activity, it should be possible to restore epidermal specification by adding back the lost factor. Expression of either a dominant-negative form of the activin receptor or the activin-binding protein follistatin neuralizes ectodermal explants^{5,7}, pointing to the growth factor activin, or a close relative, as the epidermal inducer. To test this hypothesis, we cultured ectoderm in dispersal for several hours in the presence of various concentrations of activin. The results of such an experiment are shown in Fig. 1. At low concentrations of activin, cells subjected to prolonged dispersal expressed the neural marker NCAM¹⁰, as

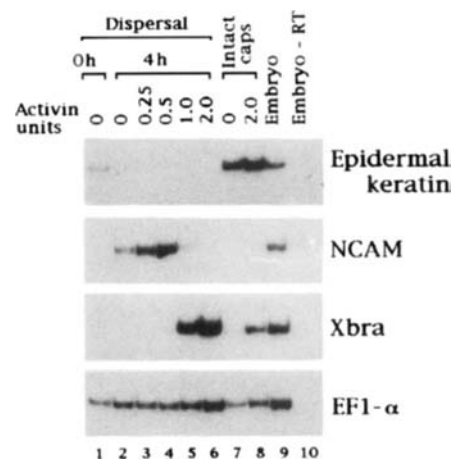
in the absence of activin. As expected from earlier work¹¹, higher concentrations of activin induced the dispersed ectoderm to form mesoderm. At no concentration was the epidermis-specific keratin XK81 (ref. 12) expressed. Even when activin concentrations separated by only 25% were used, no epidermis was formed at any dose (results not shown). Activin is able to inhibit the neuralization of dispersed cells, but only by inducing mesoderm, not epidermis.

Many members of the TGF- β family of secreted growth factors, to which activin belongs, have been found in *Xenopus* embryos, including several of the bone morphogenetic proteins or BMPs¹³. In particular, Bmp-4 is strongly expressed in the gastrula ectoderm^{8,9}. We find that this factor, in contrast to activin, is a potent inducer of epidermis and inhibitor of auto-neuralization (Fig. 2a). Dissociated animal caps cultured in the presence of Bmp-4 form epidermis after reaggregation and culture, expressing epidermal keratin. NCAM, expressed after prolonged dispersal in the absence of added factors, is suppressed. The response to inducer concentration is graded: expression of epidermal keratin increases with Bmp-4 concentration. A second marker of epidermal differentiation, the surface antigen Epi-1 (ref. 14), is also induced by Bmp-4 (Fig. 2c). As reported elsewhere^{9,15}, Bmp-4 induces the mesodermal marker Xbra in intact caps, which form epidermis in the absence of added factors, but only at concentrations of BMP far higher than those required to induce epidermis in dissociated ectoderm (Fig. 2b). Thus Bmp-4 can apparently substitute for intercellular signals lost when gastrula ectoderm is cultured in dispersion, suppressing autoneuralization and restoring ectodermal cells to the epidermal fate of intact explants. The two activities of Bmp-4—neural suppression and epidermal induction—always occur together, in this and subsequent experiments, leading us to conclude that they represent a single action.

A dominant-negative type II activin receptor (Δ IXAR1)^{5,6} and the activin antagonist follistatin⁷ neuralize explanted ectoderm. To determine whether these reagents might act by antagonizing Bmp-4, we investigated whether each could block the effect of Bmp-4 on dispersed cells. Figure 3a demonstrates that the truncated receptor can block epidermalization by Bmp-4: cells from control caps are induced to express epidermal keratin (lane 3), but those from embryos expressing Δ IXAR1 are not (lane 6). These latter cells express NCAM despite exposure to Bmp-4 protein. This result is consistent with the recent finding that Δ IXAR1 can block induction of ventral mesoderm by Bmp-

FIG. 1 Activin does not induce epidermis. Late-blastula animal caps were disaggregated, cultured for 4 h in various concentrations of activin, and reaggregated. RNA was extracted for reverse transcription-polymerase chain reaction (RT-PCR) at the end of neurulation. In the absence of activin and at low activin concentration, dispersed cells were neuralized, expressing the general neural marker NCAM¹⁰ (lanes 2–4). At higher activin concentrations mesoderm was induced (lanes 5–6), as shown by the presence of Xbra message²⁷. At no concentration was epidermis formed. Cells that were reaggregated immediately after dispersal went on to express the epidermis-specific cytokeratin XK81 (ref. 12) (lane 1), as did intact caps, which also expressed Xbra when cultured in activin (lane 8).

METHODS. Animal caps were dissociated by rinsing in calcium- and magnesium-free medium (CMFM²⁸) and gentle swirling. Outer, epithelial layers, which are not easily dissociated, were stripped away and discarded. Dispersed cells were cultured in 1 $\times$ calcium- and magnesium-free modified Barth's solution (identical to CMFM but with 5 mM HEPES substituted for Tris). In experiments in which purified growth factors were used, 0.5 mg ml⁻¹ BSA was added to all culture media. Cells were reaggregated by transfer to 0.75 $\times$ modified Ringer's (MR) solution and swirling; reaggregates contained cells from about six animal caps. For details of embryological procedures see ref. 29. RT-PCR was also performed as in ref. 29, with the modification that random hexamers rather than oligo(dT) were used to prime reverse transcription. The ubiquitous message EF1- α is used to monitor RNA recovery³⁰. Sibling control embryos served as a positive control (lane 9), and PCR on a sample of the same RNA that had not been reverse-transcribed was a control for



RT-PCR contamination (lane 10). Epidermal keratin PCR oligonucleotide sequences were: upstream, 5'-CACCAGAACACAGAGTAC-3', downstream, 5'-CAACCTTCCCATCAACCA-3'; other oligonucleotide sequences can be found in ref. 29. The activin used in this experiment was human recombinant activin A; units are arbitrary.

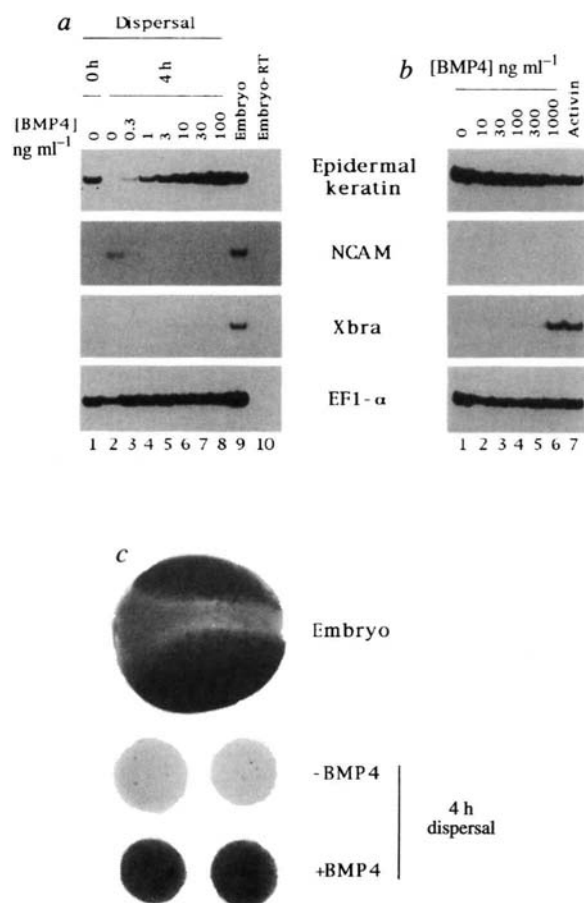


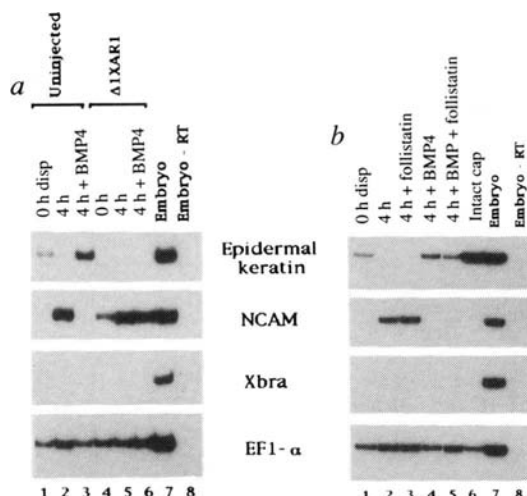
FIG. 2 Bmp-4 induces epidermis in dispersed ectoderm. Dissociated stage-9 animal cap cells (a, c) as well as intact caps (b) were incubated for 4 h with or without Bmp-4 protein. In the absence of added factor, dispersed cells expressed NCAM after reaggregation and culture to stage 18 (a, lane 2). In contrast, dispersed cells exposed to Bmp-4 expressed epidermal keratin, with expression increasing with Bmp-4 dose (lanes 3–8). NCAM expression was suppressed at even the lowest doses of inducer. The mesodermal marker Xbra was not induced in dispersed cells by Bmp-4 at the concentrations used here. Intact caps were mesodermalized by a high concentration of Bmp-4 (b). At $1 \mu\text{g ml}^{-1}$, Bmp-4 induced Xbra expression in intact caps (b, lane 6). Activin also induced Xbra in intact caps. Epidermal keratin was expressed by intact caps at all inducer concentrations. The epidermis-specific antigen Epi-1 (ref. 14) is induced by Bmp-4 treatment of dispersed ectoderm (c). As previously reported¹⁴, Epi-1 was expressed on the entire epidermal surface of the late neurula embryo, but completely excluded from the neural plate (c, top). This antigen was almost entirely absent from reagggregates of inner-layer animal-cap cells cultured in dispersion for 4 h from late blastula stages (middle). However, cells exposed to Bmp-4 (50 ng ml^{-1}) during dispersed culture expressed Epi-1 after reaggregation and culture (bottom). The antigen was not present on all cells, but appeared on a subset of cells throughout the spherical aggregates. Samples were fixed for 15 min in 15% trichloroacetic acid at 4°C and stored in methanol; immunohistochemistry was otherwise performed as in reference 6.

4 in whole caps⁹. This mutant receptor has also been reported to block the activity of Vg-1, another member of the TGF- β family¹⁶. Thus $\Delta 1\text{XAR1}$ can interfere with signalling by growth factors other than activin; it is not known whether the wild-type receptor transduces signals from these ligands. The effect of follistatin was first examined using soluble protein. As Fig. 3b shows, follistatin protein does not block Bmp-4 in the dispersed cell assay, although the same preparation of follistatin, at a 100-fold lower concentration, completely inhibits activin (results not shown). In other experiments, we find that even a 50-fold molar excess of follistatin protein does not prevent induction of epidermis by Bmp-4. We have also been unable to block the effect of Bmp-4 on dispersed cells with injected RNA for follistatin (results not shown), although injected animal caps

are neuralized, as previously reported⁷, and do not form mesoderm in response to activin.

Xenopus ectoderm cultured in dispersion during early gastrula stages form neural tissue, implying that individual ectoderm cells are neurally specified at the start of gastrulation. But intact animal caps explanted at this stage develop as epidermis. It follows that intact ectoderm contains an activity responsible for imposing and maintaining epidermal fate, an activity that is lost, presumably by dilution, when the cells are dispersed. We now report that the soluble growth factor Bmp-4 can substitute for this lost activity, inducing epidermis in dispersed cells and suppressing autoneuralization. Bmp-4 can exert this effect at low concentrations, below 1 ng ml^{-1} or about 40 pM. The pattern of Bmp-4 expression is also consistent with a function in the induction of

FIG. 3 A dominant-negative activin receptor, but not follistatin protein, can block the induction of epidermis by Bmp-4. Two-cell embryos were injected with 3 ng of RNA encoding the truncated activin receptor ($\Delta 1\text{XAR1}$) (ref. 5). Animal caps from injected and uninjected embryos were cut and dissociated at the late blastula stage. Dispersed blastomeres were incubated for 4 h in the presence or absence of 50 ng ml^{-1} Bmp-4 protein, reagggregated and cultured to late neurula stage. Bmp-4 suppressed neural development and induced epidermis in dispersed ectoderm from control embryos (a, lane 3), but not in cells from injected embryos, which continued to express NCAM strongly (lane 6). Follistatin protein could not block the epidermalizing activity of Bmp-4 (b). Dispersed late-blastula ectoderm was cultured in medium containing 50 ng ml^{-1} Bmp-4 protein alone, $1 \mu\text{g ml}^{-1}$ follistatin protein alone, both proteins at these concentrations, or in the absence of either factor. Prolonged dispersal led reagggregated ectoderm to express NCAM (b, lane 2); exposure to follistatin alone had no effect (lane 3). Bmp-4 suppressed NCAM and induced epidermal keratin (lane 4). Follistatin protein, even at a high dose, is unable to block epidermalization by Bmp-4 (lane 5), although, in accompanying controls, this protein was highly active against activin (not shown).



epidermis and the suppression of neural development. *In situ* hybridization shows that RNA for Bmp-4 is present in the entire animal cap at the start of gastrulation, as well as in the ventral and lateral marginal zone^{8,9}. At later stages, the transcript disappears from the portion of the ectoderm that becomes the neural plate⁹. Although our experiments cannot establish definitively that Bmp-4 is the endogenous epidermal inducer, the demonstration that epidermis can be induced at all is significant, because this has not previously been reported in any vertebrate embryo.

If Bmp-4 or a related substance acts within the gastrula animal cap to impose an epidermal fate, how does neural tissue form? Dispersion experiments imply that a block of epidermal induction may be sufficient. We have shown that the neuralizing effect of the truncated activin receptor can be attributed to interference with Bmp-4 signalling. However, the activin-binding protein follistatin, which also neuralizes *Xenopus* ectoderm when supplied as RNA⁷, does not block Bmp-4 in our assay. These observations present a paradox, to which we can envisage two possible resolutions. It is possible that the endogenous epidermal inducer is not Bmp-4 but a related molecule, which can be bound by follistatin. Alternatively, injection of follistatin RNA may prevent epidermal induction indirectly, by interfering with an early signalling process. Several molecules in addition to follistatin have recently been shown to neuralize *Xenopus* ectoderm^{17–20}. It will be interesting to see whether these factors, and the endogenous signals from Spemann's organizer, act by antagonizing Bmp-4 in some way.

Our proposal that Bmp-4 acts within the ectoderm to induce epidermis and suppress neural development suggests a close relationship to mesodermal patterning in the marginal zone, and perhaps to early patterning in the fly embryo as well. Experiments involving overexpression of Bmp-4 or of a dominant-negative BMP receptor^{15,21} argue that this factor acts within the marginal zone to ventralize the mesoderm and to suppress dorsal specification⁸ (reviewed in ref. 22). Thus it seems that Bmp-4 may play a similar role in the marginal zone and the animal cap. The secreted protein noggin, expressed in Spemann's organizer, has been shown to neuralize ectoderm and also to dorsalize ventral mesoderm^{18,23}; perhaps a capacity to antagonize Bmp-4 in some way could underlie both activities. The notion that dorsal-ventral patterning has a common basis in ectoderm and mesoderm was proposed years ago by Yamada²⁴. In the *Drosophila* embryo, the boundary between the neurogenic and dorsal epidermis-forming regions of the blastoderm is thought to be established by the product of the *dpp* gene, a Bmp-4 homologue. In particular, *dpp* can induce epidermis in regions of the ectoderm that would be neurogenic in its absence^{25,26}.

We have shown that the secreted growth factor Bmp-4 can induce epidermis in dispersed gastrula ectoderm, suppressing neural fate. This is the first report of an epidermal inducer in early vertebrate development. Bmp-4 is expressed in the gastrula ectoderm, at the time that fate is decided, leading us to propose that Bmp-4 acts endogenously to specify epidermis, and that neural induction may involve a BMP antagonist. □

19. Rao, Y. *Genes Dev.* **8**, 939–947 (1994).
20. Taira, M., Otani, H., Saint-Jeannet, J.-P. & Dawid, I. B. *Nature* **372**, 677–679 (1994).
21. Suzuki, A. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 10255–10259 (1994).
22. Harland, R. M. *Proc. natn. Acad. Sci. U.S.A.* **91**, 10243–10246 (1994).
23. Smith, W. C., Knecht, A. K., Wu, M. & Harland, R. M. *Nature* **361**, 547–549 (1993).
24. Yamada, T. *Biol. Bull.* **98**, 98–121 (1950).
25. Ferguson, E. A. & Anderson, K. V. *Cell* **71**, 451–461 (1992).
26. Wharton, K. A., Ray, R. P. & Gelbart, W. M. *Development* **117**, 807–822 (1993).
27. Smith, J. C., Price, B. M., Green, J. B. A., Weigel, D. & Herrmann, B. G. *Cell* **67**, 79–87 (1991).
28. Sargent, T. D., Jamrich, M. & Dawid, I. B. *Dev. Biol.* **114**, 238–246 (1986).
29. Wilson, P. A. & Melton, D. A. *Curr. Biol.* **4**, 676–686 (1994).
30. Krieg, P., Varnum, S., Wormington, M. & Melton, D. A. *Dev. Biol.* **133**, 93–100 (1989).

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Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*

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IN *Drosophila* the amount of neurogenic ectoderm, from which the central nervous system (CNS) derives, is regulated by a dorsal-ventral system of positional information in which two secreted molecules of antagonistic functions, decapentaplegic (*dpp*) and short-gastrulation (*sog*), play fundamental roles^{1–4}. The vertebrate homologue of *dpp* is either *bmp-4* or *bmp-2* (ref. 5), and the homologue of *sog* is *chd*^{4,6,7} (*s-chordin*). In *Xenopus* the CNS is induced by signals emanating from the organizer⁸, and two proteins secreted by the organizer, noggin⁹ and follistatin¹⁰, have been shown to induce neural tissue in animal-cap assays. Here we report that Chd, another organizer-specific secreted factor⁶, has neuralizing activity and that this activity can be antagonized by Bmp-4. Inhibition of the function of the endogenous Bmp-4 present in the animal cap¹¹ also leads to neural differentiation. We suggest that conserved molecular mechanisms involving *chd/sog* and *bmp-4/dpp* gene products pattern the ectoderm in *Xenopus* and in *Drosophila*.

The induction of neural differentiation was assayed by the expression of N-CAM RNA, a pan-neural marker (neurons and glia), or of NF-M (neurofilament-M) RNA, a pan-neuronal marker (neurons only), in injected animal caps explanted at early gastrula stage and cultured until siblings reached the tailbud stage (stage 27). Control injections of β -galactosidase (β -gal) messenger RNA or DNA construct did not change the differentiation of the animal caps (Fig. 1a, lanes 2 and 3), which form atypical epidermis^{9,10}. Injection of *chd* mRNA induced the expression of N-CAM and NF-M to levels comparable to those found in intact embryos of the same stage (Fig. 1a, lane 4). To test whether Chd would also be active at gastrulation¹², when neural induction normally occurs, we injected cytomegalovirus (CMV)-promoter-driven DNA, which was as active in neurogenesis as *chd* mRNA (Fig. 1a, lane 5). To test whether this property might be conserved in its *Drosophila* homologue⁴, *sog* mRNA was injected and similar results obtained (Fig. 1a, lane

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1. Spemann, H. & Mangold, H. *Arch. mikrosk. Anat. EntwMech.* **100**, 599–638 (1924).
2. Hamburger, V. *The Heritage of Experimental Embryology: Hans Spemann and the Organizer* (Oxford Univ. Press, New York, 1988).
3. Grunz, H. & Tacke, L. *Cell. Differ. Dev.* **28**, 211–218 (1989).
4. Godsave, S. F. & Slack, J. M. W. *Dev. Biol.* **134**, 486–490 (1989).
5. Hemmati-Brivanlou, A. & Melton, D. A. *Nature* **359**, 609–614 (1992).
6. Hemmati-Brivanlou, A. & Melton, D. A. *Cell* **77**, 273–281 (1994).
7. Hemmati-Brivanlou, A., Kelly, O. G. & Melton, D. A. *Cell* **77**, 283–295 (1994).
8. Fainsod, A., Steinbeisser, H. & De Robertis, E. M. *EMBO J.* **13**, 5015–5025 (1994).
9. Hemmati-Brivanlou, A. & Thomsen, G. *Dev. Genet.* (in the press).
10. Kintner, C. R. & Melton, D. A. *Development* **99**, 311–325 (1987).
11. Green, J. B. A. & Smith, J. C. *Nature* **347**, 391–394 (1990).
12. Jonas, E., Sargent, T. D. & Dawid, I. B. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5413–5417 (1985).
13. Kingsley, D. M. *Genes Dev.* **8**, 133–146 (1994).
14. Akers, R. A., Phillips, C. R. & Wessels, N. K. *Science* **231**, 613–616 (1986).
15. Graff, J., Thies, R. S., Song, J. J., Celeste, A. J. & Melton, D. A. *Cell* **79**, 169–179 (1994).
16. Schulte-Merker, S., Smith, J. C. & Dale, L. *EMBO J.* **13**, 3533–3541 (1994).
17. Witta, S. E., Agarwal, V. R. & Sato, S. M. *Development* **121**, 721–730 (1995).
18. Lamb, T. M. et al. *Science* **262**, 713–718 (1993).

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6). Neural differentiation by *Chd* took place in the absence of mesoderm induction, as demonstrated by the lack of expression of α -actin RNA at the tail-bud stage (Fig. 1a) and of the pan-mesodermal marker *Xbra* and the dorsal mesoderm markers *gsc*, *noggin* and *folliculin* at the gastrula stage (Fig. 1b). *Chd* induced visible cement glands, which are the most anterior ectodermal structures in *Xenopus*, as confirmed by the molecular marker CG13 (Fig. 1c). The neural tissue induced was of the type present in the anterior CNS, as documented by the expression of the anterior neural markers *XANF-1*, *XIF3*, *Otx-2* and some *En-2*, and by the lack of induction of the posterior neural marker *XIHbox 6* (Fig. 1c). Histologically (Fig. 2h) this neural tissue is of what embryologists called the archencephalic (forebrain) type⁸. The floor-plate marker *F-spondin*¹³ was not induced (Fig. 1c). We conclude that *Chd*, the *Xenopus* homologue of *sog*, has a potent neuralizing activity. Like *noggin*⁹ and *folliculin*¹⁰, *Chd* induces neural tissue of the anterior type.

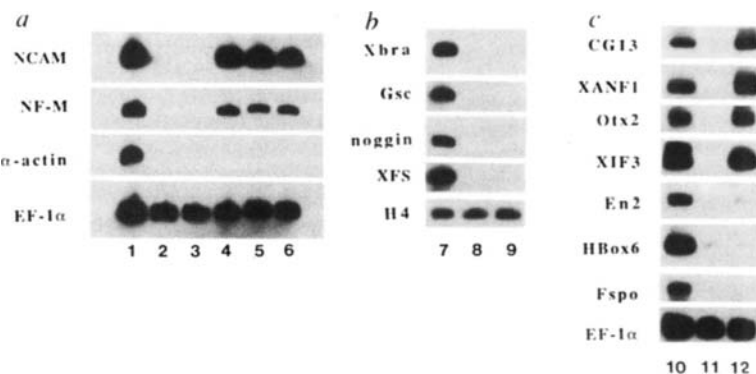
We were prompted to study the role of *chd* in neural induction by its pattern of expression in the mesodermal mantle at the late-gastrula stage (Fig. 2a, b), in which *chd* expression foreshadows the shape of the neural plate that will appear in the overlying ectoderm (Fig. 2c). In *Drosophila*, genetic studies have shown that *dpp* antagonizes *sog* *in vivo*^{2,3,4}, and this led us to test whether Bmp-4 could antagonize *Chd* in *Xenopus* pattern formation. Injection of *chd* mRNA resulted in dorsialized *Xenopus* embryos (Fig. 2d, e). Injection of Bmp-4 DNA (under the control of the cytoskeletal actin promoter, CSKA, whose expression does not start until early gastrulation⁹) into adjoining vegetal blastomeres resulted in the rescue of the dorsialized pattern and the formation of rather normal trunk-tail structures (Fig. 2f). In this experiment Bmp-4 and *chd* were injected into different cells and because a CSKA promoter construct and synthetic mRNA were used, the signals should act non-cell-autonomously and antagonize each other after the gene products were constitutively expressed. The effectiveness of Bmp-4 when introduced as a DNA construct is in keeping with the view that Bmp-4

functions as a ventralizing agent during gastrulation^{14,15}. Having found that Bmp-4 can act as an antagonist of *Chd* in mesodermal patterning, we next tested whether it would affect its neural-inducing activity.

Histological analysis showed that the induction of cement gland and of solid masses of neural tissue by *chd* mRNA in animal caps can be eliminated by co-injection of CSKA-Bmp-4 DNA (Fig. 2g-i). The same antineurogenic action of Bmp-4 was observed when a marker of mature neurons, β -tubulin type II¹⁶, was used as a molecular probe in *in situ* hybridizations (Fig. 2j-l). CSKA-Bmp-4 DNA was also able to inhibit the neural induction caused by expressing *chd* from a CMV promoter (Fig. 3a, lanes 4 and 5). We conclude that Bmp-4 expressed at the gastrula stage has a strong antineurogenic activity in gain-of-function experiments.

The animal cap (as well as the ventrolateral marginal zone) has significant amounts of zygotic Bmp-4 transcripts at the early gastrula stage¹¹. To test whether these endogenous transcripts might prevent neural differentiation in the animal cap, directing the tissue towards a more ventral epidermal fate, we pursued a loss-of-function approach. Injection of mRNA encoding a dominant-negative Bmp-2/4 receptor^{17,18} induced the neural markers N-CAM and NF-M and, as expected in case of a block in the Bmp-4 signalling pathway, this could not be reversed by co-injection of Bmp-4 DNA (Fig. 3b, lanes 6 and 7). The dominant-negative receptor interacts not only with Bmp-4, but also with Bmp-2 (refs 17, 18) and perhaps with other related factors¹⁹. To determine whether Bmp-4 itself was responsible for the antineurogenic activity in the animal cap, we used antisense RNA. It has recently been shown that antisense Bmp-4 can dorselize *Xenopus* ventral mesoderm and that the antisense approach works in *Xenopus*, at least for genes expressed early after mid-blastula (H.S., A. Fainsod, C. Niehrs, Y.S. and E.M.D.R., manuscript submitted). When full-length antisense Bmp-4 RNA was injected, neural markers were induced, and this effect, unlike that of the dominant-negative receptor, could be reversed by co-

FIG. 1 *Chd* can induce anterior neural markers in the absence of mesoderm induction. Animal caps were excised from injected embryos at stage 10, cultured until stage 27 (a, c) or stage 11 (b), and assayed for the expression of marker mRNAs. Lanes contained: 1, RNA extracted from whole embryos at stage 27; 2, embryos injected with β -gal mRNA⁴; 3, with pCMV- β -gal DNA¹²; 4, *chd* mRNA⁶; 5, pCMV-*chd* (constructed for this study); 6, *sog* mRNA⁴; 7, activin-treated stage 11 animal caps⁶; 8, control β -gal mRNA; 9, *chd* mRNA; 10, whole-embryo RNA at stage 27; 11, control β -gal mRNA; 12, *chd* mRNA. Note that *chd* mRNA, *chd* DNA and *sog* mRNA induce the neural markers N-CAM and NF-M²⁶ without increasing the mesodermal marker α -actin. *Xbra*, *gsc*, *noggin* and *folliculin* (XFS) are not induced by *chd* mRNA injection. *chd* mRNA, but not control β -gal mRNA, induces cement gland (CG13 (ref. 27)) and anterior neural markers (*XANF-1* (ref. 28), *Otx-2*, *XIF3* (ref. 26) and some *En-2*) but not the posterior marker *XIHbox 6* (*Hoxb9*). The floorplate marker *F-spondin* (*Fspo*) is not induced. Elongation factor 1 α (*EF-1 α*) and histone H4 were used as RNA-loading controls. METHODS. For all RNAs 0.3 ng, and for the CMV plasmids 0.1 ng, were injected per blastomere. Embryos were injected into each animal blastomere at the 8-cell stage. Animal caps were explanted at the gastrula stage and cultured in 0.3 $\times$ modified Barth's solution until siblings reached the desired stage⁶. Only healthy animal caps in which cells continued to divide were used for further analysis. Total RNA was isolated by using the RNA-STAT kit⁶, using two extractions to reduce contamination by genomic DNA. For each set of primers the number of cycles required for the linear range in the reverse transcription polymerase chain reaction (RT-PCR) was determined empirically. Many of the primer sets, conditions for PCR and the number of cycles were as described (H.S., A. Fainsod, C. Niehrs, Y.S. and E.M.D.R., submitted, and refs. 10, 11, 28), but the following were new. CG13, (forward primer (F), 5'-AGTTGTTGATTTGTGAAACC; reverse primer (R), 5'-CTTCTCTAAATCAACACAGG; 28 cycles); *Otx-2* (accession no. U19813) (F, 5'-

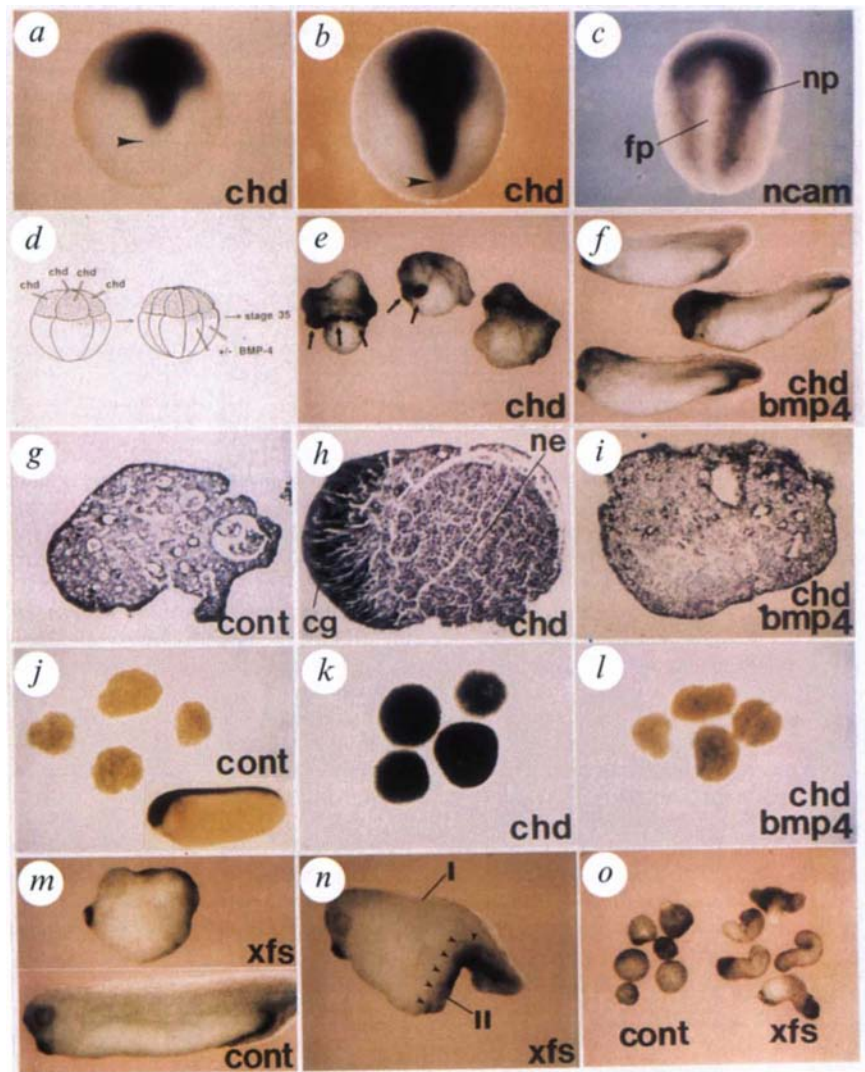


GGATGGATTGTTGCACAGTC; R, 5'-CACTCTCCGAGCTCACTTCTC; 30 cycles); NF-M (accession no. M25696) (F, 5'-GCGGGTACCTTCTAAT-AGTCAC; R, 5'-GGCTTGGCTGTGGTTCTGAAGG; 28 cycles); *XIF3* (F, 5'-ATCCTCCAGGCTATCCACCTCC; R, 5'-TAGCGGACCTTCTCTATGAAGC; 28 cycles); *F-spondin* (F, 5'-TCTGGCAGTATGGCAACGTC; R, 5'-GTA-CAATGCTCGCCTTGAGTCTC; 30 cycles); *noggin* (F, 5'-ATGAT-CATTCACAGTGCCCTGTGAC; R, 5'-AGATTAGTCCAAGAGTCTCAGCATGAGC; 30 cycles); *folliculin* (F, 5'-ATGGTAAATGAAAGGATCCAGCCGGGCATG; R, 5'-ATTCACCTACAGTTGCAAGATCCACTGTG; 30 cycles). The DNA expression construct pCMV-*chd* was constructed by subcloning a *HindIII*-*XbaI* fragment of pSP35-*chd* (ref. 6) into pCDM8 (Invitrogen). Previous studies¹² have shown that a similar construct (pCMV- β -gal) accumulates a significant amount of the protein product only after gastrulation starts. mRNAs were synthesized with SP6 polymerase by using the 'Message Machine' kit (Ambion); all synthetic mRNAs used in this study were engineered so that they contained 5' leader and 3' trailer sequences derived from the *Xenopus* β -globin mRNA^{4,6}.

injection of CSKA-Bmp-4 DNA (Fig. 3b, lanes 8 and 9). As specificity controls, we used Δ Bmp-4 antisense RNA lacking the mature carboxy-terminal region that is conserved among TGF- β growth factors, which behaved as its full-length antisense counterpart (lane 10); Δ Bmp-2 antisense RNA, which had no neural inducing activity (lane 11); and, as a negative control, *gsc* antisense RNA, which is not expressed in animal caps and, as expected, had no effect (lane 12). We conclude from these loss-of-function experiments that Bmp-4, but not Bmp-2, is required in the animal cap to prevent neural differentiation.

The genes *chd*, *noggin* and *folistatin* are expressed in the organizer, induce neural tissue of the archencephalic type and can dorsalize mesoderm. A dorsalizing function for follistatin has not been noted before, but as shown here *folistatin* mRNA, like *noggin* and *chd*, is able to induce dorsalization, partial secondary axes and the formation of dorsal tissues in ventral marginal zone (VMZ) explants in microinjected *Xenopus* embryos (Fig. 2m-o). This supports the view²⁰ that follistatin might not bind exclusively activin, and indicates that follistatin can counteract ventral signals. Because of these common features, we tested whether the antineurogenic activity of Bmp-

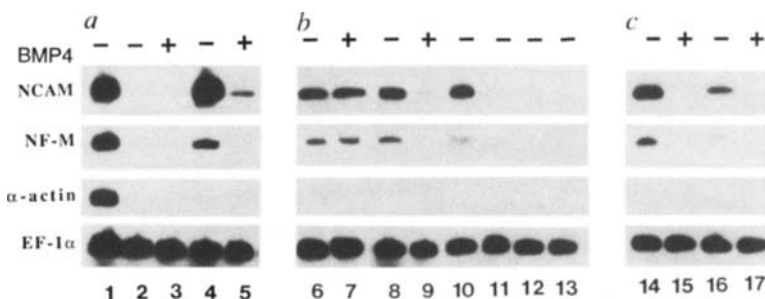
FIG. 2 *chd* mRNA is expressed at the right time and place to act as a neural inducer, and its effects can be antagonized by injected Bmp-4 DNA. *a*, *In situ* hybridization of *chd* mRNA at late gastrula (stage 12); expression in the mesoderm foreshadows the shape of the future neural plate, which will appear later in the ectoderm. *b*, At early neurula (stage 13) *chd* expression in the mesodermal layer extends more posteriorly, reaching the closing blastopore (arrowhead) and starts converging towards the midline. *c*, N-CAM hybridization *in situ* demarcates the neural plate (np) in the ectoderm of a stage-14 *Xenopus* neurula; the prospective floorplate does not express N-CAM (fp). The shape of the neural plate resembles that of *chd* expression in the mesoderm. *d*, Experimental design used to test the antagonism between *Chd* and Bmp-4 in patterning the marginal zone. *chd* mRNA was injected into the four animal blastomeres at the 8-cell stage, and at the 16-cell stage two adjoining vegetal blastomeres were injected with a control plasmid or with Bmp-4 DNA. *e*, Embryos injected with *chd* mRNA and control pCSKA- β -gal plasmid; note that the embryos are dorsalized⁶ (dorsoanterior index (DAI) 7.3, $n=15$) and have expanded cement glands (arrows) and short trunk-tail structures. *f*, Embryos injected radially with *chd* as in *e*, but also pCSKA-Bmp-4 DNA; the dorsalized phenotype has been rescued¹¹ and that the resulting embryos have normal trunk-tail structures (DAI 5.7, $n=18$; data from two independent experiments). *g*, Histological appearance of animal caps injected with control β -gal mRNA; this morphology corresponds to atypical epidermis. *h*, After injection with *chd* mRNA together with control pCSKA- β -gal plasmid, a large mass of neural tissue (small basophilic cells with prominent nuclei, ne) as well as cement gland tissue (large ectodermal vacuolated cells containing melanin pigment, cg). *i*, Co-injection of pCSKA-Bmp-4 DNA together with *chd* mRNA eliminates neural and cement gland differentiation. *j*, Control animal caps injected with β -gal mRNA and hybridized *in situ* with the neuronal marker β -tubulin II. No signal is seen in the animal caps; inset shows expression of the marker¹⁶ in tailbud embryos. *k*, Expression of β -tubulin indicates differentiation of neurons after injection of *chd* mRNA together with control pCSKA- β -gal DNA. *l*, Differentiation of neurons is suppressed by injection of pCSKA-Bmp-4 DNA together with *chd* mRNA. *m*, Embryo with enhanced dorso-anterior structures after injection of XFS mRNA into the upper region of the four vegetal blastomeres at the 8-cell stage; a normal embryo (cont) is shown as well. If injected at the 2- or 4-cell stage, XFS mRNA interferes with gastrulation, which is probably why its dorsalizing activity has not been recognized before. *n*, Embryo with a partial secondary (II) axis (indicated by arrowheads) after injection of a single ventral-vegetal blastomere with XFS mRNA at the 16-cell stage; 23% of such embryos were dorsalized and 54% had secondary axes ($n=26$). Antibody markers showed no secondary notochords and histological sections showed secondary neural tubes and somites. *o*, VMZs injected with β -gal mRNA



(control, cont) or with XFS mRNA; the explants with follistatin elongate. METHODS. *chd* and β -gal mRNA were injected at 0.3 (*d-f*) or at 0.4 (*f-l*) ng per blastomere. pCSKA- β -gal and pCSKA-Bmp-4 DNAs were injected at 0.15 ng (*d-f*) or 0.1 ng (*g-l*) per blastomere. Whole-mount *in situ* hybridizations were according to Harland's method with minor modifications⁶. The N-CAM probe was made by PCR amplification of a complementary DNA fragment (nucleotides 260-1,790 in Genbank M25696) and subcloning into the *Xho*I and *Eco*RI sites of pBluescript KS(-); antisense N-CAM probe was made by linearizing with *Xho*I and transcribing with T7 polymerase. The antisense *chd* probe has been described⁶ and the β -tubulin II probe was obtained by linearizing plasmid 24-10²⁶ with *Not*I and transcribing with T3 RNA polymerase. XFS mRNA was injected at about 0.1 ng per blastomere; two independent experiments gave similar results.

FIG. 3 Bmp-4 expression counteracts neural induction by *chd* DNA and antisense Bmp-4 RNA but not neurogenesis by dominant-negative Bmp-2/4 receptor. Lanes: 1, whole-embryo RNA at tailbud stages; 2, animal caps injected with control pCMV- β -gal DNA together with pCSKA- β -gal DNA; 3, control pCMV- β -gal DNA and pCSKA-Bmp-4 DNA; 4, pCMV-*chd* DNA co-injected with control pCSKA- β -gal DNA; 5, pCMV-*chd* co-injected with pCSKA-Bmp-4 DNA; 6, dominant-negative Bmp-2/4 receptor (DNBMMPR) mRNA and pCSKA- β -gal DNA; 7, DNBMMPR mRNA and pCSKA-Bmp-4 DNA (note that Bmp-4 has no effect); 8, full-length antisense Bmp-4 RNA and control plasmid; 9, antisense Bmp-4 RNA and pCSKA-Bmp-4 DNA; 10, truncated Δ Bmp-4 antisense RNA from which the mature growth factor portion was deleted is still able to induce neural differentiation; 11, Δ Bmp-2 antisense RNA (lacking the growth factor region) does not induce neural differentiation; 12, control antisense *gsc* mRNA, which is not expressed in the animal cap; 13, antisense β -gal RNA; 14, *noggin* mRNA and control pCSKA- β -gal; 15, *noggin* mRNA and pCSKA-Bmp4 DNA, showing that Bmp-4 counteracts neurogenesis by *noggin*; 16, *folistatin* mRNA (*XFS*) and control plasmid DNA; 17, *XFS* mRNA and pCSKA-Bmp-4 DNA.

METHODS. Embryo manipulations and RNA analyses were as in Fig. 1. For CMV-*chd*, pCMV- β -gal, pCSKA- β -gal and pCSKA-Bmp-4 DNAs, 0.1 ng per blastomere were injected; for DNBMMPR, 0.5 ng; for antisense



Bmp-4, antisense Δ Bmp-4, antisense Δ Bmp-2, antisense *gsc* and antisense β -gal RNAs, 0.6 ng; for *noggin* mRNA, 0.1 ng; and for *XFS* mRNA, 0.15 ng. All antisense RNAs were as described (H.S., A. Fainsod, C. Niehrs, Y.S. and E.M.D.R., submitted). To obtain pSP35-DNBMMPR, cDNA encoding residues 1–174 of Bmp-2/4 receptor¹⁷ (followed by a termination codon) was amplified by PCR and subcloned into the *EcoRI*-*Sall* sites of the mRNA expression vector⁶. For pSP35-*noggin* and pSP35-*folistatin* the entire coding sequence was amplified by PCR, adding *NcoI* and *XbaI* restriction sites. To make synthetic mRNA, vectors were linearized with *EcoRI*, except for pSP35-DNBMMPR, which was linearized with *PstI*, and transcribed with SP6 RNA polymerase.

4 might inhibit neural differentiation caused by *noggin* and *folistatin* mRNAs. Interestingly, CSKA-Bmp-4 blocked neural induction by *noggin* and *folistatin* (Fig. 3c, lanes 14–17).

This raises the question of whether any differences exist in the function of *chd*, *noggin* and *folistatin*. With *chd* and *Bmp-4*, there is good genetic evidence linking their *Drosophila* homologues, *sog* and *dpp*, to the formation of CNS^{1,4}. No *Drosophila* homologues have been reported for *noggin* and *folistatin*. A particularly interesting observation is provided by *lim-1*, an organizer-specific homeobox gene that in mouse is required for the formation of all brain structures anterior to rhombomere 3 (ref. 21). *Xenopus* cells injected with activated *Xlim-1* mRNA are able to induce neural tissue in neighbouring cells in the absence of *noggin* or *folistatin* expression²², but do express *chd* mRNA (M. Taira and I. Dawid, personal communication). Dominant-negative receptors can interact with multiple signalling pathways¹⁹, and it may be worthwhile to explore in future whether the neuralizing effects reported for activin receptor mutants^{23,24} are mediated by a block of Bmp-4 signalling.

In this study we have shown that *Chd*, like its homologue *sog*, leads to the formation of neural tissue and that Bmp-4 can antagonize this effect. Bmp-4 transcripts present endogenously in the animal cap are required to prevent neural differentiation

in *Xenopus* explants. An antagonism between *Chd* and Bmp-4 is consistent with the roles of their homologues in *Drosophila*. On the other hand, the inhibition by Bmp-4 of the neural induction caused by *noggin* and *folistatin* is not easily explained. One hypothesis that may help reconcile the data is that, in both *Drosophila* and vertebrates, neural induction may be specified by the dorsal-ventral positional values of the ectoderm. In *Xenopus* ventral values would be induced by high concentrations of Bmp-4 (and presumably other factors) and dorsal values by antagonistic signals emanating from the organizer. It is not known how *noggin*, *folistatin* and *Chd* function from a biochemical viewpoint; for example, they may exert their activity through their own receptor-mediated pathway, affect Bmp-4 processing or bind directly to Bmp-4 or other ventralizing factors. However, the three molecules are able to dorsalize *Xenopus* embryos. Thus, the same dorsal-ventral patterning signals are used by the mesoderm and by the ectoderm. A similar situation has been reported recently in *Drosophila*, although in this case the ectoderm is the sole source of the signal²⁵. Finally, it is conceivable that the dorsal-ventral system is involved only in the specification of the most anterior (archencephalic) neural tissue, and that the key to the differentiation of the posterior CNS will be found in an independent anterior-posterior patterning system. □

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1. Ferguson, E. L. & Anderson, K. V. *Cell* **71**, 451–461 (1992).
2. Ferguson, E. L. & Anderson, K. V. *Development* **114**, 583–597 (1992).
3. François, V., Solloway, M., O'Neill, J. W., Energy, J. & Bier, E. *Genes Dev.* **8**, 2602–2616 (1994).
4. Holley, S. A. et al. *Nature* **376**, 249–253 (1995).
5. Padgett, R. W., St Johnson, R. D. & Gelbart, W. M. *Proc. natn. Acad. Sci. U.S.A.* **90**, 2905–2909 (1994).
6. Sasai, Y. et al. *Cell* **79**, 779–790 (1994).
7. François, V. & Bier, E. *Cell* **80**, 19–20 (1995).
8. Hamburger, V. *The Heritage of Experimental Embryology: Hans Spemann and the Organizer*. (Oxford Univ. Press, New York, 1988).
9. Lamb, T. M. et al. *Science* **262**, 713–718 (1993).
10. Hemmati-Birvanlou, A., Kelly, O. G. & Melton, D. A. *Cell* **77**, 283–295 (1994).
11. Fainsod, A., Steinbeisser, H. & De Robertis, E. M. *EMBO J.* **13**, 5015–5025 (1994).
12. Niehrs, C., Keller, R., Cho, K. W. Y. & De Robertis, E. M. *Cell* **72**, 491–503 (1993).
13. Ruiz i Altaba, A., Cox, C., Jessell, T. M. & Kilar, A. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8268–8272 (1993).
14. Dale, L., Howes, G., Price, B. M. J. & Smith, J. C. *Development* **115**, 573–585 (1992).
15. Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E. & Hogan, B. L. M. *Development* **115**, 639–647 (1992).

16. Richter, K., Grunz, H. & Dawid, I. B. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8086–8090 (1988).
17. Graff, J. M., Thies, S. R., Song, J. J., Celeste, A. J. & Melton, D. A. *Cell* **79**, 169–179 (1994).
18. Suzuki, A. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 10255–10259 (1994).
19. Schulte-Merker, S., Smith, J. C. & Dale, L. *EMBO J.* **13**, 3533–3541 (1994).
20. Matzuk, M. M. et al. *Nature* **374**, 360–363 (1995).
21. Shawlot, W. & Behringer, R. R. *Nature* **374**, 425–430 (1995).
22. Taira, M., Otani, H., Saint-Jannet, J. P. & Dawid, I. B. *Nature* **372**, 677–699 (1994).
23. Hemmati-Birvanlou, A. & Melton, D. A. *Nature* **359**, 609–614 (1992).
24. Hemmati-Birvanlou, A. & Melton, D. A. *Cell* **77**, 273–281 (1994).
25. Frasch, M. *Nature* **374**, 464–467 (1995).
26. Sharpe, C. R., Pluck, A. & Gurdon, J. B. *Development* **107**, 701–714 (1989).
27. Jamrich, M. & Sato, S. *Development* **105**, 779–786 (1989).
28. Zaraisky, A. G. et al. *Dev. Biol.* **152**, 373–382 (1992).

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Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor

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SINCE the discovery that epidermal growth factor (EGF) can accelerate opening of the eyelids¹, the EGF receptor (EGF-R) has been extensively studied and is now considered to be a prototype tyrosine kinase receptor². Binding of EGF or of transforming growth factor- α (TGF- α) or other related factors activates the receptor and induces cell proliferation and differentiation. Although it is not found on haematopoietic cells, the EGF-R is widely expressed in mammals and has been implicated in various stages of embryonic development³. Here we investigate the developmental and physiological roles of this receptor and its ligands by inactivating the gene encoding EGF-R. We find that EGF-R^{-/-} mice survive for up to 8 days after birth and suffer from impaired epithelial development in several organs, including skin, lung and gastrointestinal tract.

The EGF-R gene was inactivated in exon 2, which encodes an amino-terminal segment of the EGF-R (Fig. 1a). The weight and fertility of EGF-R^{+/-} animals were normal. Whereas 23% (37 of 162) of the embryos at embryonic days (E) 12–17 were homozygous for the disrupted gene, only 13% (65 of 546) of the newborn mice were EGF-R^{-/-}. This discrepancy was due to embryonic lethality as early as E10, as illustrated by genotyped resorption sites (Fig. 1b). No messenger RNA corresponding to exons 1 and 2 (spanning the disruption site) was detected using reverse transcription with the polymerase chain reaction (RT-PCR; data not shown) and no EGF-R protein was detected in fibroblasts from EGF-R^{-/-} embryos (Fig. 1c). Although the EGF-R^{-/-} embryos appeared normal at E10–17, half were smaller, with proportionately smaller placentas (data not shown). This growth retardation is consistent with the suggested role of EGF-R in signalling in feto-placental interactions⁴. For example, human intrauterine growth retardation correlates with decreased expression and phosphorylation of placental EGF-R⁴, and depletion of salivary EGF or administration of EGF-R antiserum decreases the number and size of the offspring in mice⁵.

All EGF-R^{-/-} mice were born with open eyes, had short curly whiskers, and died within 8 days (Fig. 1d). The lens was prolapsed and adhered to the cornea (data not shown). This eye phenotype allowed a reliable identification of EGF-R^{-/-} mice as early as E17. By day 2 their eyelids closed, covering the eyes as in normal littermates. Normally the eyelids close at E16 and open again at 2 weeks of age, a process accelerated by EGF¹. Thus, eyelid development was delayed in EGF-R^{-/-} mice. Newborn EGF-R^{-/-} mice were classified into three groups: (1) runt pups (0.4–0.5 g; control littermates, 1.5 g) which died or were eaten immediately after birth; (2) small pups (0.8 g as opposed to 1.5 g), which often had gasping breathing and lived up to 2 days; and (3) normal-sized pups (1.2–1.5 g) which thrived for 3–4 days, but then lost weight with dehydration and malnutrition and died at day 7–8 (final weight, 2 g compared

with 6 g) (Fig. 1d). EGF-R^{-/-} mice also had a rapid heart rate and a one-day delay in external ear opening. About one third of the smallest mice had cleft palates (data not shown). Congenital hypothyroidism was excluded as the cause of growth retardation on the basis of serum thyroxine measurements (data not shown).

All homozygous newborn mice had thin skin, often almost transparent, with haematomas and petechiae. The skins of EGF-R^{-/-} animals gradually became dry and flaky (Fig. 1d). No hair outgrowth was seen by day 7, when control littermates already had a thick fur coat. EGF-R^{-/-} mice had short curly whiskers at birth, whereas those of control littermates were long and straight (Fig. 1d). The epidermis of newborn EGF-R^{-/-} mice was thinner and contained fewer hair follicles than weight-matched littermates, resembling the skin of normal E17 embryos and reflecting immature development (Fig. 2a–c). By day 2, the EGF-R^{-/-} epidermis had an organized basal layer, but the nuclear layer was one cell layer thicker and the stratum granulosum was more prominent than in control skin. Six-day skin was stained with antibodies for different stages of epidermal differentiation: keratin 5 (basal layer), keratin 10 (suprabasal layer), filaggrin (stratum corneum) and loricrin (stratum granulosum). Whereas no major differences were seen in keratin 5 and 10 immunostaining, EGF-R^{-/-} skin stained weakly with anti-filaggrin (data not shown) and contained less loricrin (Fig. 2h, i), suggesting disturbed terminal differentiation of the EGF-R^{-/-} epidermis and hair follicles. Hair follicles were disoriented and few hairs grew through the epidermis (Fig. 2d–g). EGF-R^{-/-} mice had a much more severe hair phenotype than *waved-2* mice, which have attenuated EGF-R signalling⁶ owing to a mutated cytoplasmic domain. Apart from wavy hair and impaired lactation, these mice develop normally^{6,7}. Similarly, TGF- α ^{-/-} and *waved-1* mice, which express less TGF- α ^{8,9}, also have disorganized follicles. Taken together, these findings suggest that several EGF-R ligands are involved in hair growth and support a role for the EGF-R in follicle differentiation.

All EGF-R^{-/-} mice exhibited an intestinal phenotype of varying severity. Although small 2-day-old survivors could feed and had milk in their stomachs, their intestines were haemorrhagic and distended. In contrast, the intestines from normal-sized newborn EGF-R^{-/-} mice appeared to be healthy at birth, but had less twisted loops, shorter and fewer villi, and a thinner muscle layer than control littermates (Fig. 3a–d). Whereas the intestines of both normal and EGF-R^{-/-} newborn mice stained positively for alkaline phosphatase, an enterocyte-differentiation marker, and had all the differentiated cell types, EGF-R^{-/-} intestines were less developed, with fewer and shorter villi. The epithelium of the EGF-R^{-/-} tongue also appeared to be immature and was thinner and less organized, with no fungiform taste papillae (Fig. 3g, h). Furthermore, EGF-R deficiency also resulted in decreased proliferation of jejunal enterocytes, as determined by bromodeoxyuridine labelling (data not shown). The delayed development of EGF-R^{-/-} villi was already apparent at E14.5, when villi normally start forming (Fig. 3i, j).

By day 7, the surviving EGF-R^{-/-} animals were markedly undernourished. Their stomachs were devoid of milk and the proximal parts of their intestines were fragile and haemorrhagic. At the end stage, fluid and free gas were detectable in the abdominal cavity among dilated intestinal loops, although no perforations or pneumatosis intestini were seen. Histological analysis showed that the intestinal villi had completely disintegrated and contained dilated vessels with abundant haemorrhage (Fig. 3e, f). The dark-coloured intestinal loops, fluid in the abdominal cavity and the histological phenotype resembled necrotizing enterocolitis, a human disease associated with premature birth. The gradual destruction of villi in EGF-R^{-/-} mice may have resulted from mucosal damage by milk, combined with deficient digestive capacity and impaired regeneration and proliferation from the crypts. The gastrointestinal phenotype is consistent with the expression of both EGF-R and its ligands in intestinal mucosa¹⁰ and the stimulatory effect of EGF on proliferation and

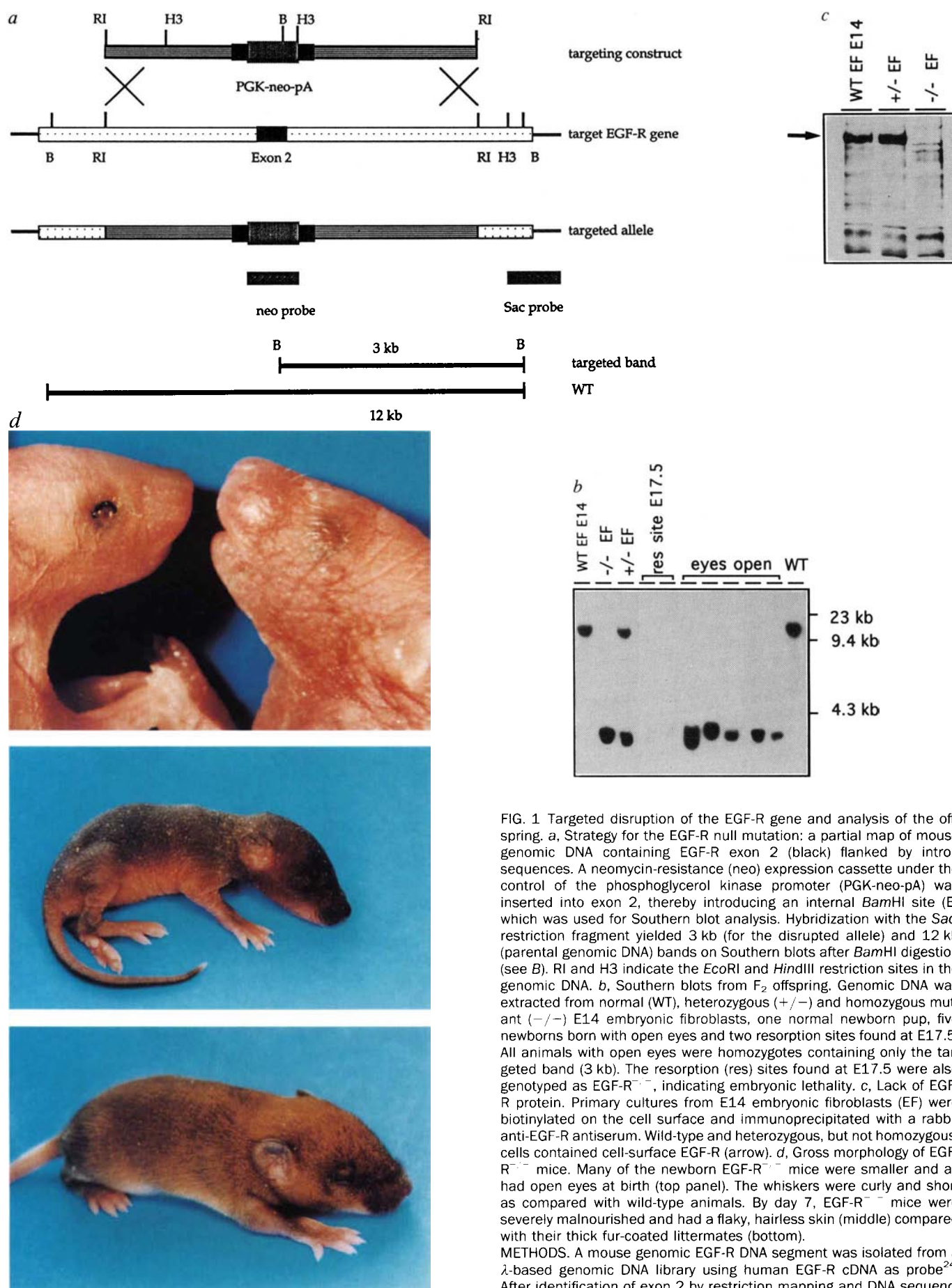
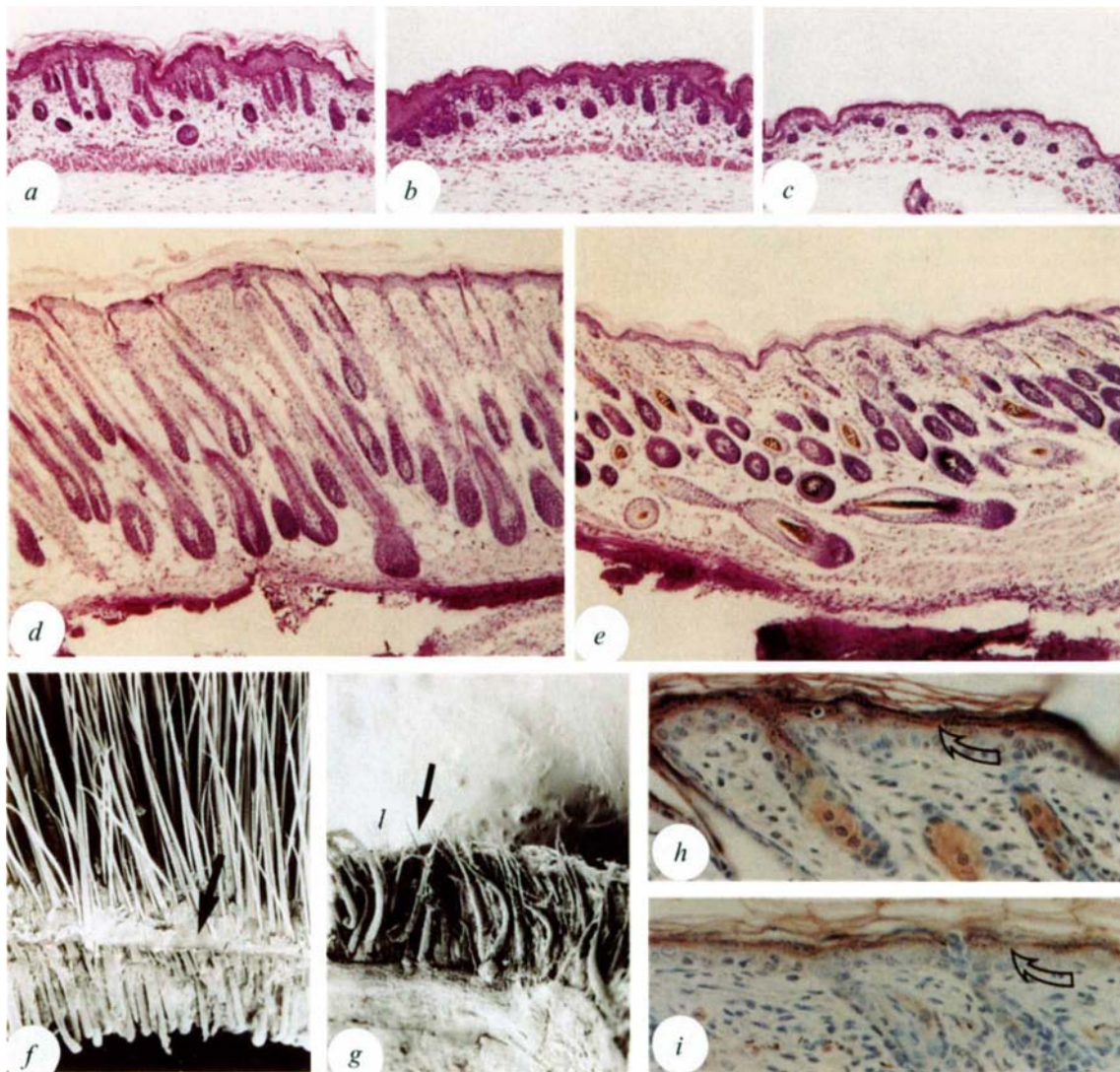


FIG. 1 Targeted disruption of the EGF-R gene and analysis of the offspring. **a**, Strategy for the EGF-R null mutation: a partial map of mouse genomic DNA containing EGF-R exon 2 (black) flanked by intron sequences. A neomycin-resistance (neo) expression cassette under the control of the phosphoglycerol kinase promoter (PGK-neo-pA) was inserted into exon 2, thereby introducing an internal *Bam*HI site (B) which was used for Southern blot analysis. Hybridization with the *Sac*I restriction fragment yielded 3 kb (for the disrupted allele) and 12 kb (parental genomic DNA) bands on Southern blots after *Bam*HI digestion (see **B**). RI and H3 indicate the *Eco*RI and *Hind*III restriction sites in the genomic DNA. **b**, Southern blots from F₂ offspring. Genomic DNA was extracted from normal (WT), heterozygous (+/-) and homozygous mutant (-/-) E14 embryonic fibroblasts, one normal newborn pup, five newborns born with open eyes and two resorption sites found at E17.5. All animals with open eyes were homozygotes containing only the targeted band (3 kb). The resorption (res) sites found at E17.5 were also genotyped as EGF-R^{-/-}, indicating embryonic lethality. **c**, Lack of EGF-R protein. Primary cultures from E14 embryonic fibroblasts (EF) were biotinylated on the cell surface and immunoprecipitated with a rabbit anti-EGF-R antiserum. Wild-type and heterozygous, but not homozygous, cells contained cell-surface EGF-R (arrow). **d**, Gross morphology of EGF-R^{-/-} mice. Many of the newborn EGF-R^{-/-} mice were smaller and all had open eyes at birth (top panel). The whiskers were curly and short as compared with wild-type animals. By day 7, EGF-R^{-/-} mice were severely malnourished and had a flaky, hairless skin (middle) compared with their thick fur-coated littermates (bottom).

METHODS. A mouse genomic EGF-R DNA segment was isolated from a λ -based genomic DNA library using human EGF-R cDNA as probe²⁴. After identification of exon 2 by restriction mapping and DNA sequenc-

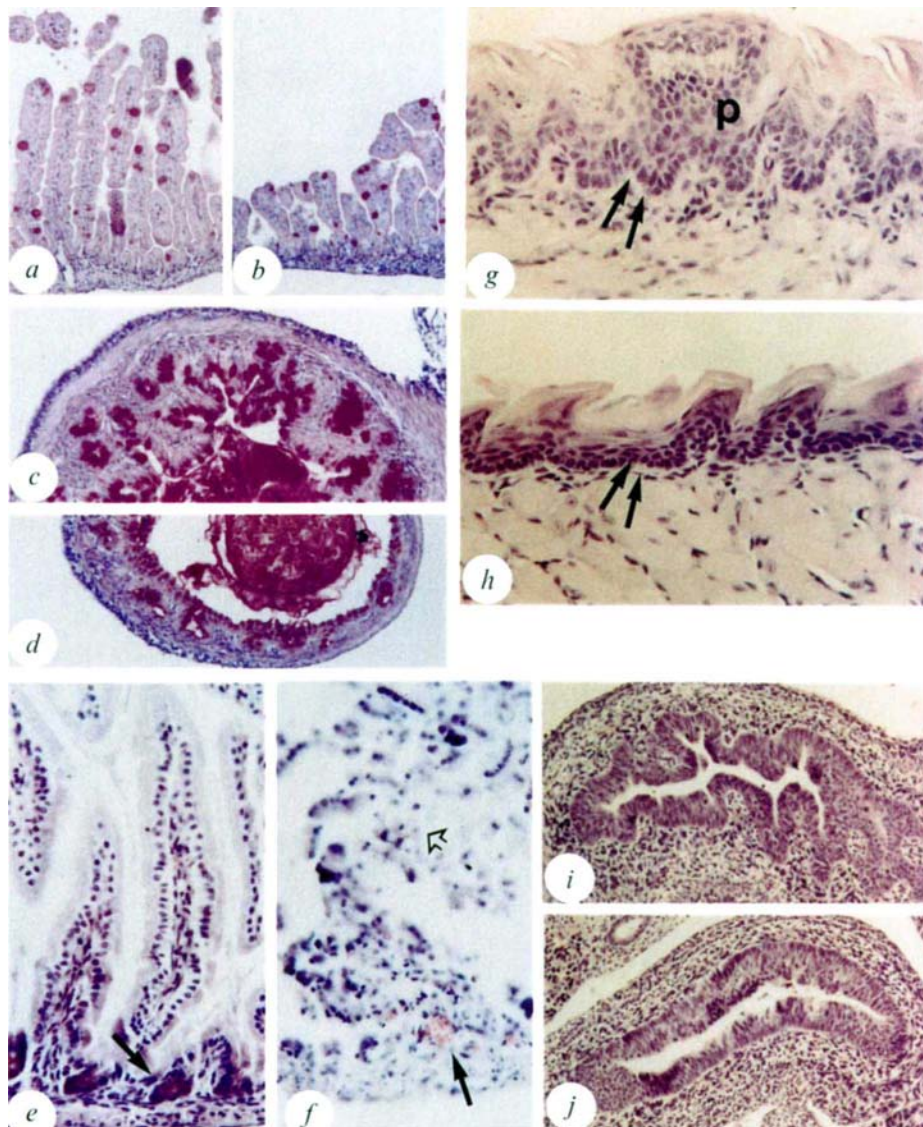


▲ FIG. 2 Histological analysis of the skin. In newborn animals, the control skin had more hair follicles and the epidermis was thicker (a) when compared with the more immature state seen in EGF-R^{-/-} skin (b and c; backskin of two different mice). At postnatal day 6, control animals had long, straight hair follicles (d, f), whereas the follicles of EGF-R^{-/-} animals were disorganized and seldom reached the stratum corneum (e, g), as was also evident from scanning electron microscopy (f, g). The arrows in f and g indicate the surface of the skin. Immunostaining showed a stronger reactivity for loricrin in the stratum granulosum (open arrow) of 6-day-old control animals (h) than in EGF-R^{-/-} mice (i). Haematoxylin–eosin staining in a–e; scale bar, 100 µm in a–g and 25 µm in h, i.

METHODS. Animals were killed by decapitation. Tissues were fixed in 4% paraformaldehyde and processed into paraffin sections by routine procedures. They were stained with haematoxylin–eosin or with rabbit anti-loricrin as described³⁰. The specific immunoreactivities were detected using peroxidase-conjugated donkey anti-rabbit immunoglobulins (Amersham) and diaminobenzidine as chromogen (Sigma). For scanning electron microscopy, the skin was fixed overnight in 2% glutaraldehyde (T. Pella), washed in PBS, dehydrated in a series of ethanols and processed for electron microscopy using routine procedures. Samples were analysed in a JEOL 840 scanning electron microscope.

ing, a *NotI* site that was preceded by a stop codon in frame was introduced by PCR-based mutagenesis following the codon for amino acid 57 (ref. 6). A *NotI* cassette for the neomycin-resistance gene was inserted into the *NotI* site under the PGK promoter²⁵. The targeting vector also contained an HSV-thymidine kinase cassette²⁶ downstream from the disrupted genomic fragment. The targeting vector was introduced to JM-1 embryonic stem cells (129SV/J) (J.M. and R.A.P., unpublished data) by electroporation. Genomic DNA isolated from embryonic stem cell clones after neomycin-positive (G418-resistant; 300 µg ml⁻¹) and thymidine-kinase-negative (FIAU-resistant; 1 µM; gift from Bristol Meyer Squibb) selections was digested with *Bam*HI and analysed by Southern blotting using digoxigenin-labelled probes²⁷. Four clones in which one allele of the EGF-R gene was disrupted were injected into C57BL/6J blastocysts. Chimaeric males derived from three different ES cell clones were mated with Swiss Webster Black females. Animals bearing the targeted EGF-R allele were interbred and the F₂ offspring analysed further by Southern blotting. On a few occasions, F₁ animals were derived from matings between the chimaeras and C57BL/6J females, but no difference was noted between their F₂ offspring and the Swiss Webster Black F₂ mice. The methodology used to generate the EGF-R^{-/-} mice was based on standard protocols²⁸. Embryonic fibroblast cultures²⁹ from wild-type, heterozygous and homozygous EGF-R fetuses were grown to confluence and biotinylated with NHS-biotin³⁰. Biotinylated proteins were immunoprecipitated with rabbit anti-mouse EGF-R and analysed using reducing conditions in 7% SDS-polyacrylamide gel electrophoresis. After electrotransfer to nitrocellulose, biotinylated proteins were detected using streptavidin-conjugated peroxidase and chemiluminescence³⁰.

FIG. 3 Histological analysis of the intestines. Comparison between newborn control and EGF-R^{-/-} mice shows the shorter villi in jejunum (normal in a, and EGF-R^{-/-} in b) and the flatter mucosa in colon (normal in c, and EGF-R^{-/-} in d). Periodic acid-Schiff staining shows the bright pink mucus-containing goblet cells (a-d). Occasionally a pseudostratified epithelium was visible in the intervillous space of the intestines of the EGF-R^{-/-} mice, suggestive of an immature epithelial state. Before death at postnatal day 8, jejunal villi from EGF-R^{-/-} mice (f) had totally disintegrated, with cell debris filling the lumen (open arrow), and capillaries were enlarged (arrow). In contrast, the control animal (e) had well formed, long villi and Paneth cells in the crypts (arrow). The tongue from a 6-day-old control animal (g) had a well organized basal layer in the epithelium (arrows) and a fungiform papilla (p), whereas the epithelium in the EGF-R^{-/-} mouse (h) was immature. At E14, the jejunal mucosa showed developing villi in normal (i), but not in EGF-R^{-/-} (j) littermates of the same size. Phloxine tartrazine staining (scale bar in e, f, 25 μ m). Haematoxylin-eosin staining; scale bar, 25 μ m (a, b, g, h) and 50 μ m (c, d, i, j). Tissues were fixed and processed as for Fig. 2.



differentiation of enterocytes^{11,12}. Milk, especially colostrum, contains high levels of EGF and TGF- α , which is thought to stimulate proliferation and maturation of the gastrointestinal tract of newborn animals or infants^{13,14}.

Consistent with their breathing difficulties, the lungs of many small EGF-R^{-/-} mice were dramatically different from controls (Fig. 4a, b). These were condensed, with collapsed alveoli close to the pleural surface, or had dilated terminal bronchi closer to the pleura (Fig. 4c-e). Only a few cells in the collapsed alveoli stained for surfactant SP-C or SP-A (data not shown). In contrast to the thin alveolar epithelium of control mice, the alveolar septae of EGF-R^{-/-} mice were thicker and more cellular (Fig. 4f, g). The breathing problems of many EGF-R^{-/-} pups could be correlated with the severity and extent of collapsed alveoli.

The immaturity in lung development resembles neonatal respiratory distress syndrome. In humans, respiratory failure results from insufficient production of surfactant, which is required for the alveoli to remain open, combined with immaturity of other structural elements such as alveoli and airways¹⁵. In mice, surfactant is produced by differentiated type-II pneumocytes from E15 onwards¹⁶. EGF enhances pneumocyte differentiation¹⁷, whereas antiserum against EGF blocks their maturation¹⁸. Furthermore, EGF enhances branching and stimulates proliferation in fetal lung cultures¹⁹ and accelerates lung maturation in fetal rabbits²⁰. Infusion of EGF *in utero*

into fetal lambs and rhesus monkeys rescues prematurely born animals from respiratory distress syndrome^{21,22}, whereas EGF autoantibodies induce it²³. Taken together, the immature lung phenotype of EGF-R^{-/-} mice resembles human neonatal respiratory distress syndrome and suggests a role for the EGF-R in lung development.

The common denominator for the abnormalities in EGF-R^{-/-} mice was defective or delayed epithelial development. This phenotype was not merely a consequence of growth retardation, because the analyses were done using weight-matched control animals. Furthermore, runted pups from normal mice do not have these developmental problems. In lungs, this resulted in respiratory failure, which was the apparent cause of death soon after birth. In the intestine, the epithelial immaturity led to wasting, followed by a necrotizing enterocolitis-like disorder. These mice died presumably of septic shock by cardiac and respiratory failure. Impaired epithelial development was probably the basis for the open-eye phenotype at birth. Whether the epithelial phenotype resulted from decreased cell proliferation or defective differentiation is as yet unclear. The latter possibility is supported by the lower expression of loricrin in EGF-R^{-/-} skin, and was suggested by the EGF-induced precocious eyelid-opening and incisor eruption¹. The former possibility is, however, supported by the decreased cell proliferation in EGF-R^{-/-} intestinal mucosa.

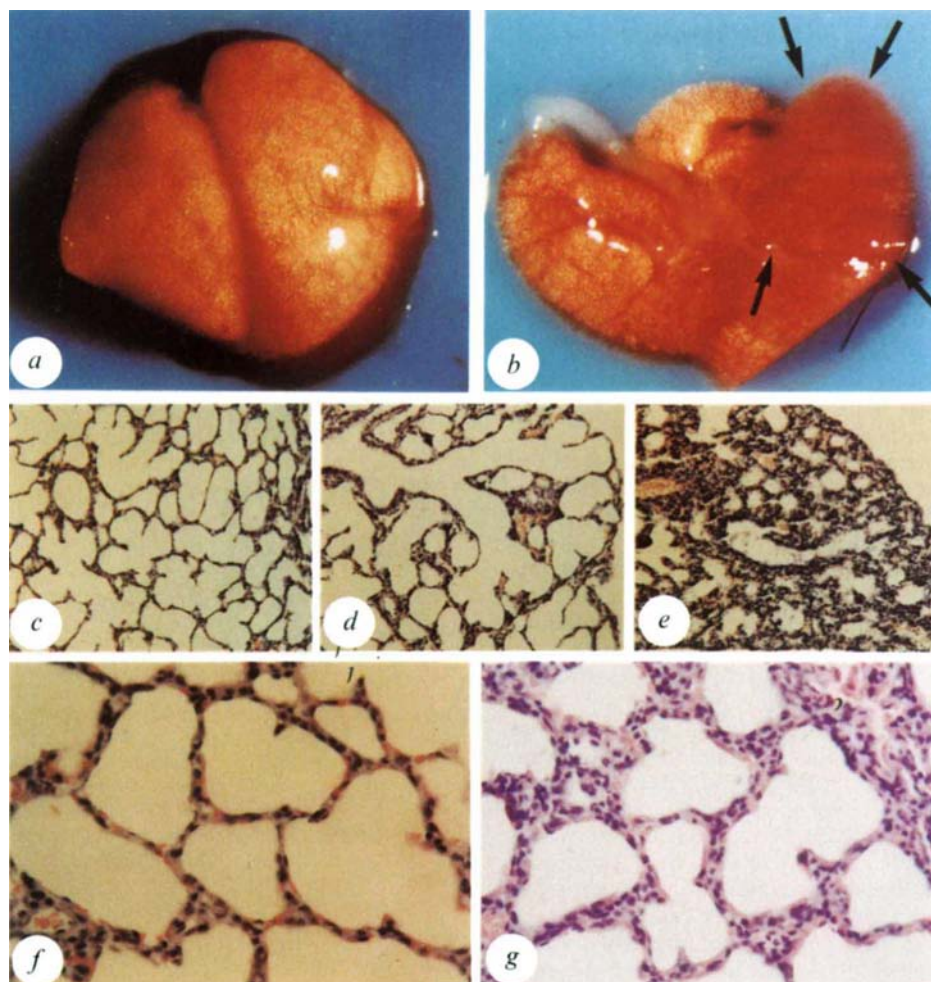


FIG. 4 Lung morphology and histology. Macroscopically, a newborn control lung (a) had a smooth surface full of air-filled alveoli, whereas the EGF-R^{-/-} lung (b) was atelectic, with airless lung parenchyma (arrows), or it had many more visible bullae on its surface. Histological examination of the lungs from newborn mice showed symmetrical alveoli in a control mouse (c), whereas in the EGF-R^{-/-} animal, large

airways were close to the pleural surface (d), and collapsed, poorly air-filled alveoli (e) were visible. Furthermore, the terminal bronchi were closer to the pleura than in normal lungs. At postnatal day 4, the control alveoli had a thin wall (f) compared with thick-walled EGF-R^{-/-} alveoli (g). Haematoxylin-eosin staining; scale bar, 50 μ m (c–e) and 25 μ m (f, g). Tissues were fixed and processed as for Fig. 2.

In conclusion, EGF-R^{-/-} mice show growth retardation and epithelial immaturity and dysfunction, which results in gastrointestinal and lung phenotypes resembling human diseases associated with premature birth. Besides the phenotypes of the organs illustrated, other epithelia are also affected.

Note added in proof: We have now obtained several EGF-R^{-/-} mice that lived up to days 15–18. EGF-R^{-/-} mice have also been generated by Threadgill *et al.*³¹ and Sibilias and Wagner³² showing peri-implantation, mid-gestational and postnatal lethality dependent on the genetic background. □

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- Cohen, S. J. *biol. Chem.* **237**, 1555–1562 (1962).
- Schlessinger, J. & Ullrich, A. *Neuron* **9**, 383–391 (1992).
- Paria, B. C., Das, S. K., Andrews, G. K. & Dey, S. K. *Proc. natn. Acad. Sci. U.S.A.* **90**, 55–59 (1993).
- Fondacci, C. *et al. J. clin. Invest.* **93**, 1149–1155 (1994).
- Fujita, Y. *et al. J. clin. Endocr. Metab.* **72**, 1340–1345 (1991).
- Luetke, N. C. *et al. Genes Dev.* **8**, 399–413 (1994).
- Fowler, K. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **92**, 1465–1469 (1995).
- Luetke, N. C. *et al. Cell* **73**, 249–261 (1993).
- Mann, G. B. *et al. Cell* **73**, 249–261 (1993).
- Miettinen, P. J. *Ped. Res.* **33**, 481–486 (1993).
- Malo, C. & Ménard, D. *Gastroenterology* **83**, 28–35 (1982).
- Ménard, D., Arsenault, P. & Pothier, P. *Gastroenterology* **94**, 656–663 (1988).
- Beardmore, J. M., Lewis-Jones, D. I. & Richards, R. C. *Ped. Res.* **17**, 825–828 (1983).
- Okada, M. *et al. Life Sci.* **48**, 1151–1156 (1991).

- Jobe, A. *Ann. Medicine* **23**, 687–691 (1991).
- Glasser, S. W., Korfhagen, T. R., Bruno, M. D., Dey, C. & Whitsett, J. A. *J. biol. Chem.* **265**, 21986–21991 (1990).
- Plopper, C. G. *et al. Am. J. Physiol.* **262**, L313–L321 (1992).
- Yasui, S., Nagai, A., Oohira, A., Iwashita, M. & Konno, K. *Ped. Pulm.* **15**, 251–256 (1993).
- Warburton, D. *et al. Devl Biol.* **149**, 123–133 (1992).
- Catterton, W. Z. *et al. Ped. Res.* **13**, 104–108 (1979).
- Sundell, H. W., Gray, M. E., Serenius, F. S., Escobedo, M. B. & Stahlman, M. T. *Am. J. Path.* **100**, 707–725 (1980).
- Goetzman, B. W. *et al. Ped. Res.* **35**, 30–36 (1993).
- Raaberg, L., Nexø, E., Jorgensen, P. E., Poulsen, S. S. & Jakab, M. *Ped. Res.* **37**, 175–181 (1995).
- Derynck, R. *et al. EMBO J.* **7**, 3737–3743 (1988).
- Adra, C., Boer, P. H. & McBurney, M. W. *Gene* **60**, 65–74 (1987).
- Mansour, S. L., Thomas, K. R. & Capecci, M. R. *Nature* **336**, 348–352 (1988).
- Engler-Blum, G., Meier, M., Frank, J. & Müller, G. A. *Analyt. Biochem.* **210**, 235–244 (1993).
- Joyner, A. L. *Gene Targeting—A Practical Approach* (Oxford Univ. Press, New York, 1993).
- Hogan, B., Beddington, R., Constantini, F. & Lacy, E. *Manipulating the Mouse Embryo—A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 1994).
- Miettinen, P. J., Ebner, R., Lopez, A. R. & Derynck, R. *J. Cell Biol.* **127**, 2021–2038 (1994).
- Threadgill, D. W. *et al. Science* (in the press).
- Sibilias, M. & Wagner, E. *Science* (in the press).

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Effect of lesions in visual cortical area V4 on the recognition of transformed objects

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THE primate visual system has a remarkable capability for recognizing objects irrespective of the multitude of images they form on the retinal surface by virtue of changes in size, perspective, contrast, colour and partial obstruction by other stimuli in the visual scene. There is increasing evidence that this remarkable capacity is brought about by processes that occur earlier in the visual system than had previously been thought¹⁻¹⁴. Here I show that after ablation of area V4 in the rhesus monkey, major deficits arise in the recognition of objects that have been transformed in size, in the degree of occlusion, and in the amount of contour information provided. The ability to detect these objects when presented individually was unaffected by these lesions.

The effects of area V4 lesions were assessed using a new procedure that made it possible to alter systematically the degree to which objects are transformed by varying the relative size of the stimuli, the degree of occlusion by other stimuli, and the amount of contour information provided to make them visible. The rhesus monkeys used in this study were trained on a matching-to-sample task. A sample stimulus appeared in the centre of a colour monitor. After the stimulus had been fixated, four other objects were presented simultaneously under a variety of conditions (Fig. 1). The animal's task was to select the matching object by making a saccadic eye movement to it. Correct choices were rewarded by drops of apple juice. The animal's head was restrained and eye movements were monitored using the scleral search-coil technique¹¹. These procedures made it possible to confine selected stimuli to specific locations in the visual field. After the monkeys became proficient on the matching-to-sample tasks, which required several months of training, ~1 cm² of cortical grey matter was aspirated in area V4 (refs 11, 12). Subsequently, when the stimuli were presented, some of them fell either in the intact regions of the visual field or into those regions whose representation in area V4 had been ablated. This procedure allowed reliable, concurrent comparisons of performance in the affected and intact regions of the visual field. Two mon-

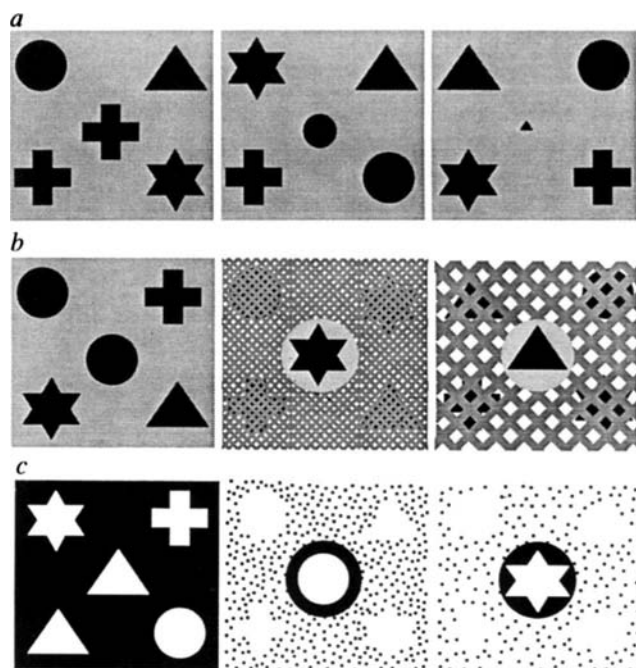
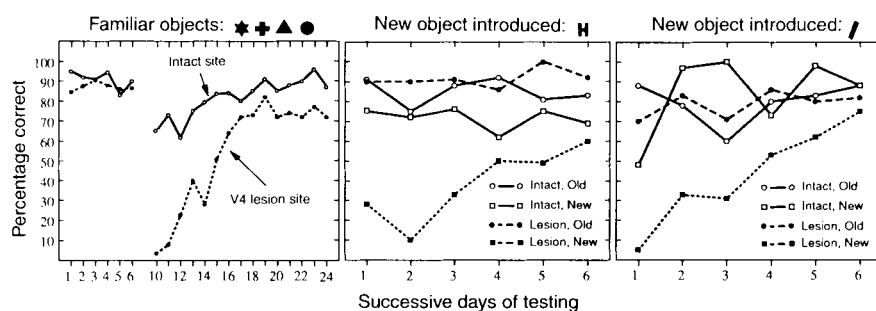


FIG. 1 Examples of the stimulus conditions used. *a*, Size series: the size of the sample object (centre stimulus) is varied randomly from trial to trial; in the left panel the sample and matching stimuli are identical in size. *b*, Occlusion series: various degrees of occlusion (centre and right panels); no occlusion (left panel). *c*, Contour series: varied amounts of contour information in (centre and right panels); full contour information (left panel).

keys were studied. In one, a unilateral lesion was made in area V4. In the other, V4 lesions were made successively in the left and right hemispheres; this animal was tested extensively after each lesion. Each animal was tested 1,000–3,000 times per day.

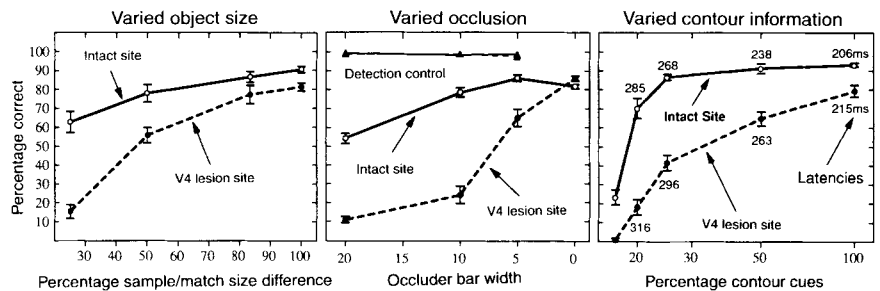
A few days after the operation, monkeys were tested with a simple detection procedure to assess their luminance and colour contrast sensitivities; after fixation a single target appeared which when correctly selected with a single saccadic eye movement led to the monkey being rewarded. The relatively mild post-operative deficits seen in the detection of low-contrast and high-spatial-frequency stimuli made it possible to map out the area in the visual field that was affected by the ablation, as had been done in an earlier extensive series of tests¹¹. The lesions eliminated a relatively large, oval portion of the contralateral lower visual field representation of area V4 with the downward

FIG. 2 Left panel: pre-operative (days 1–6) and post-operative (days 10–24) data obtained at two sites, one ablated and one preserved. Pre-operative performance was similar at the two sites. After ablation, performance deteriorated dramatically at the V4 lesion site, but then improved with repeated days of testing. Centre and right panels: performance at intact and lesion sites on successive days of testing when one new object was substituted for a familiar one after performance had asymptoted on a familiar set of figures. The graph illustrates performance with new and old objects separately. Major deficits are seen at the lesion but not at the intact site upon introduction of a new figure, followed by gradual improvement over a period of 6 successive days of testing. Performance at the lesion site remained high with the familiar figures. Each point is based on 30–120 trials. As described earlier^{11,12}, the aspiration of area V4 was carried out under general anaesthesia with the aid of a dissection microscope; ~1 cm² of cortical grey matter was removed between



the lunate and superior temporal sulci, with the lesion reaching 2–5 mm into each sulcus. The visual field region affected by the lesion was subsequently mapped out by obtaining contrast sensitivity functions with small stimuli, using the detection procedure.

FIG. 3 Performance on transformed objects. The performance is shown as percentage correct, as a function of degree of transformation for size, occlusion and contour information provided. The right panel provides latency data indicating the mean time in milliseconds required for the animal to make a saccadic eye movement to the correct target after the appearance of the matching objects. The reaction times for stimuli appearing at the lesion site were significantly longer than those made to intact sites. Each point is based on 200–500 trials. For the detection controls shown in the middle panel (triangles and thin lines) the matching stimuli were presented singly under the same display conditions. The contrast of the stimuli was between 65% and 90% relative to background and was 33% relative to the occluding arrays.



slanting long axis extending from near the fovea to an eccentricity of 8–14° of visual angle¹¹. This procedure was followed by the matching-to-sample task, examples of which appear in Fig. 1. The basic task appears in the left column. Three transformations are depicted: in the top row the size of the sample stimulus is varied; in the middle row the degree of object occlusion is varied; in the bottom row the amount of contour information provided is varied. Stimulus displays of this sort were presented in a randomized fashion within each category.

The animals were first retested after the ablation of area V4 with familiar objects; the sample stimulus presented on each trial was identical to one of the four matching stimuli subsequently presented, as shown in the left column of Fig. 1. The left panel in Fig. 2 shows data collected on successive days pre- and post-operatively in one monkey showing performance separately for two spatial locations, one whose representation in the visual system remained intact, and one whose representation had been ablated in area V4. After an initial major deficit at the lesion site, a gradual recovery occurred with repeated daily testing.

After comparable levels of performance had been reached at sites in the visual field that were intact and whose representation had been lesioned, a new figure was introduced replacing one of the familiar ones. This procedure documented the extent to which what had previously been learned could be generalized to new objects. The repeated testing that ensued revealed, as shown in the centre and right panels of Fig. 2, a major drop in performance on the new but not the old figures at the V4 lesion site. This drop recurred every time a new figure was introduced, suggesting that V4 lesions cause a significant deficit in the ability to apply to new objects what has already been learned.

Once the monkeys reached asymptotic performance with any given set of objects, their ability to recognize them under a variety of transformations was assessed. Testing each of these tasks extended over a period of several months. Figure 3 shows performance on three transformations. For each, the degree to which the objects were transformed varied randomly from trial to trial. For the size transform, the size of the sample stimulus was varied while the sizes of the matching stimuli, falling either into intact or affected regions of the visual field, were held constant. For the occlusion transform, the sample stimulus was always presented unobstructed; the degree of occlusion of the matching stimuli was varied randomly by trial. The third transformation varied the amount of contour information provided for the appearance of the matching stimuli (Fig. 1). The results for the three transformation tasks showed a major deficit in performance in the absence of area V4 (Fig. 3).

Similar results have been obtained in learning and on the transformation tasks in the second animal used in this study. For example, for the varied-occlusion condition, using the same procedures for which data are shown from the first monkey in the middle panel of Fig. 3, the percentage correct performances

in the second animal at intact sites for the same four occlusion points were 62.5%, 74.3%, 79.6% and 92.0%; for the location in the visual field where area V4 had been ablated, the performances for the corresponding points were 15.0%, 22.1%, 32.5% and 83.0% (based on 40–180 trials per data point).

In control experiments the monkeys used in this study were tested using a detection paradigm in which, after the appearance of the fixation spot, the matching stimuli shown in Fig. 1 were presented singly and appeared randomly in one of the same four positions used in the matching-to-sample task; otherwise identical display conditions were used. The results for the occlusion task are shown in the middle panel in Fig. 3 (thin lines and triangles) and show close to 100% correct detection throughout at both intact and lesioned sites. As an additional control, animals were also tested with textured arrays of the sort depicted in Fig. 4, where the task was to make a direct saccadic eye movement to the region made distinctive in appearance by either a textural difference, as shown in Fig. 4, a difference in stereoscopic depth or differential motion at the target site. With contrast levels above 18%, performance on these demanding tasks was close to 100% at both the intact and lesion-affected sites. Extensive testing showed that the percentage luminance contrasts required for 60% detection threshold at the intact and lesioned sites were 10.2% and 12.0% for monkey no. 1, and 14.6% and 15.0% for monkey no. 2.

Repeated testing with transformed objects using the matching-to-sample procedure showed considerably less recovery than was evident when the animals were studied with objects whose appearance was identical for the sample and matching stimuli, as shown in Fig. 2. Even when tested one year after the ablation, a sizeable deficit remained in the recognition of transformed



FIG. 4 Example of the type of texture array used in control studies. The target region appeared randomly in one of four positions 3.5° from the fixation point. The visual angle of the width of the lines was 3.4 arcmin.

objects. The limited recovery with transformed objects is probably due to the fact that monkeys were exposed to 16–64 different stimulus configurations in randomized presentations, whereas during the learning phase, as shown in Fig. 1, only 4 different stimuli were used, 3 of which were familiar to the animal.

Object recognition tests^{6,7} under free viewing conditions, where images impinge at many different retinal locations, yielded continued deficits with repeated testing, suggesting that there is limited spatial generalization after V4 lesions^{11,12}. Deficits in object transformations under free viewing conditions have been reported¹⁵ after inferotemporal and foveal prestriate lesions. Deficits in form recognition have also been shown^{7,16}.

The findings reported here, together with earlier reports, suggest that the extrastriate visual cortical areas of the primate, rather than each being engaged in the analysis of a singular basic visual attribute such as the processing of colour, motion, pattern or depth^{17–20}, subserve far more complex, elaborate and interactive analyses than previously realized^{1–14}. Area V4 and the regions with which it connects have now been identified as important in at least three high-level visual analyses: visual learning, the selection of non-salient objects in the visual scene, and, as shown here, the recognition of visual objects when they

undergo the great variety of image transformations encountered in life^{11,12}. □

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- Desimone, R. & Schein, S. J. *J. Neurophysiol.* **57**, 835–868 (1987).
- Felleman, D. J. & Van Essen, D. C. *Cerebral Cortex* **1**, 1–47 (1991).
- Ferrera, V. P., Neely, T. A. & Maunsell, J. H. R. *Nature* **358**, 756–758 (1992).
- Haenny, P. E., Maunsell, J. H. R. & Schiller, P. H. *Expl Brain Res.* **69**, 245–259 (1988).
- Haenny, P. E. & Schiller, P. H. *Expl Brain Res.* **69**, 225–244 (1988).
- Heywood, C. A. & Cowey, A. J. *Neurosci.* **7**, 2601–2617 (1987).
- Heywood, C. A., Gadott, A. & Cowey, A. J. *Neurosci.* **12**, 4056–4065 (1992).
- Moran, J. & Desimone, R. *Science* **229**, 782–784 (1985).
- Motter, B. C. *J. Neurosci.* **14**, 2190–2199 (1994).
- Schiller, P. H. *Behav. Brain Res.* (in the press).
- Schiller, P. H. *Visual Neurosci.* **10**, 717–746 (1993).
- Schiller, P. H. & Lee, K.-M. *Science* **251**, 1251–1253 (1991).
- Schiller, P. H. & Logothetis, N. K. *Trends Neurosci.* **13**, 392–398 (1990).
- Schiller, P. H., Logothetis, N. K. & Charles, E. R. *Visual Neurosci.* **5**, 321–346 (1990).
- Weiskrantz, L. & Saunders, R. C. *Brain* **107**, 1033–1072 (1984).
- Walsh, V., Butler, S. R., Carden, D. & Kulikowski, J. J. *Behav. Brain Res.* **50**, 115–126 (1992).
- Hubel, D. H. & Wiesel, T. N. *Nature* **225**, 41–42 (1970).
- Livingstone, M. S. & Hubel, D. H. *Science* **240**, 740–749 (1988).
- Zeki, S. M. *Brain Res.* **53**, 422–427 (1973).
- Zeki, S. M. *Nature* **284**, 412–418 (1980).

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Neural regulation of thermotaxis in *Caenorhabditis elegans*

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THERMAL stimulus is an important environmental factor influencing animal behaviour¹. However, the mechanisms underlying thermosensation and thermal adaptation are poorly understood. The nematode *Caenorhabditis elegans* can sense a range of environmental temperatures and migrate towards the cultivation temperature on a thermal gradient². This modifiable thermotactic response provides an ideal system for studying the cellular and molecular processes involved in thermosensation and thermal information storage. We have identified neurons critical for thermotaxis by killing individual cells in live animals. The results indicate that an amphid sensory neuron, AFD, is a major thermosensory neuron. Some of the genetically defined cryophilic and thermophilic mutant phenotypes were mimicked when amphid interneurons AIY and AIZ, respectively, were killed, indicating that AIY is responsible for thermophilic movement and AIZ for cryophilic movement. We propose a neural model in which regulation of the activities of the two interneurons in opposite directions, depending on the cultivation temperature, is essential for thermotaxis.

After being cultivated normally on plates seeded with bacteria (as food) and placed on unseeded plates with a thermal gradient, wild-type animals migrate to their cultivation temperature (15–25 °C) and move isothermally around that temperature² (Fig. 1a–c). These thermotactic responses are likely to be established after several critical processes are executed normally.

The AFD sensory neurons had been suggested to be involved in thermotaxis, as a *txx-1(p767)* cryophilic mutant lacked microvillus-like projections in the sensory ending of the AFD neurons but was normal for other sensory behaviours³ (Table 1). Unlike other sensory neurons in the amphid sensillum, no functional assignment has yet been made for AFD^{4–7}. We killed the AFD neurons in young larvae and assayed thermotaxis of the operated animals individually as adults by evaluating tracks of movement left on an agar surface with a thermal gradient (Fig. 1;

Table 1). When both left and right AFD neurons were killed, most of the operated animals showed cryophilic (C), athermotactic (A), or an intermediate phenotype (C/A) (Fig. 1f–h). Importantly, killing both AFDs abolished isothermal movement. These abnormalities were independent of cultivation temperatures; the phenotypes of the AFD-killed animals cultivated at 15, 20 and 25 °C were indistinguishable from one another (data not shown). When compared with phenotypic spectra of typical cryophilic, thermophilic and athermotactic mutants, the AFD-killed animals seem to mimic either cryophilic or athermotactic mutants (Table 1; I.M., unpublished results).

When a single AFD neuron (either left or right) was killed, the defects became less severe and some animals could move isothermally around their cultivation temperature (Table 1). This result implies that, although functions of both AFD neurons are necessary for normal thermotaxis, isothermal movement can be achieved without comparing informations from the left and right AFD neurons.

No significant thermotactic defects were observed when other amphid sensory neurons were killed (Table 1). In particular, killing the neuron AWA, which could send a signal to AFD by chemical synapses⁸ (Fig. 2), did not induce an abnormal phenotype (Table 1). When all amphid sensory neurons except AFD were killed, the operated animals showed normal thermotactic responses, indicating that AFD is the site of thermosensation and not a regulator of any other thermosensory neuron(s) elsewhere in the amphid sensillum.

To identify additional neurons functional for thermotaxis, the amphid interneurons which could interact with AFD were killed. AFD is electrically connected with the AIB interneurons by gap junctions, and is presynaptic to the AIY interneurons⁸ (Fig. 2). The AIB-killed animals showed no detectable abnormality in thermotaxis when compared with the spectra of wild-type animals (Table 1). The operated animals responded to different cultivation temperatures (data not shown). By contrast, a significant effect was observed when AIYs were killed (Table 1). Almost all of the AIY-killed animals exhibited clear cryophilic movement and lost the ability to move isothermally, thereby mimicking the phenotypes of several cryophilic mutants such as *txx-3(ks5)* (Table 1; Fig. 1f; I.M. and Y.O., unpublished results). One of the major postsynaptic partners of AIY is AIZ, another amphid interneuron⁸ (Fig. 2). The AIZ-killed animals mostly became thermophilic; they sought a higher temperature than their cultivation temperature and sometimes left isothermal circles on assay plates (Table 1; Fig. 1i, j). These phenotypic

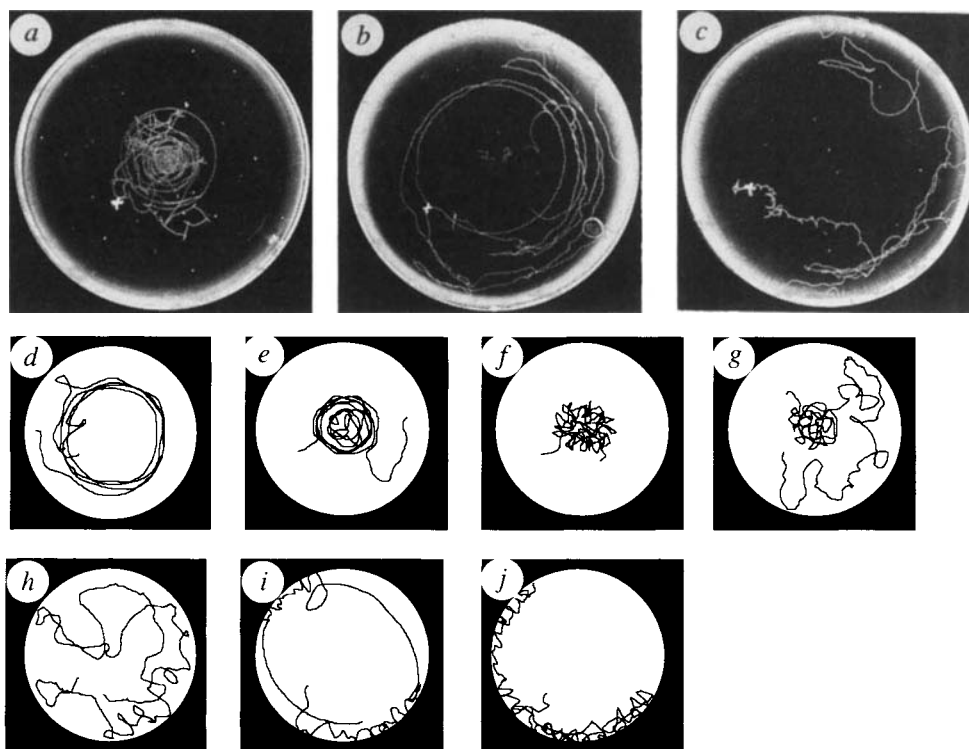
TABLE 1 Effects of laser operations on thermotaxis

Cells killed	No. of animals	No. of assays	Thermotactic phenotype							Fraction of W(+) + C(+)
			W(-)	C(+)	T(+)	T(-)	C(-)	C/A(-)	A(-)	
Amphid sensory neuron										
AFD	28	(78)	0	0	0	4	26	20	28	0.0
AFD (right)	9	(36)	8	7	0	0	14	4	3	0.42 ± 0.16
AFD (left)	9	(35)	6	7	0	1	11	5	5	0.37 ± 0.16
ADF	4	(12)	11	1	0	0	0	0	0	1.0
ASE	8	(23)	18	3	0	0	1	1	0	0.91 ± 0.12
ASH	3	(12)	8	2	0	0	1	0	1	0.83 ± 0.22
ASE + ASH	1	(4)	4	0	0	0	0	0	0	1.0
ASK + ADL + ASI	2	(7)	3	4	0	0	0	0	0	1.0
AWA	6	(19)	15	3	0	0	1	0	0	0.95 ± 0.1
All cells except AFD	2	(5)	5	0	0	0	0	0	0	1.0
Amphid interneuron										
AIB	13	(36)	29	3	0	0	1	1	2	0.89 ± 0.10
AIY	19	(44)	0	0	0	0	35	5	4	0.0
AIZ	12	(36)	2	0	17	17	0	0	0	0.06 ± 0.08
AIZ + 3 ~ 5 cells nearby	7	(20)	6	0	3	8	0	0	3	0.30 ± 0.20
Combination kill										
AFD + AIY*	4	(9)	0	0	0	2	3	1	3	0.0
AFD + AIZ	5	(16)	0	0	0	1	0	0	15	0.0
AIY + AIZ*	5	(12)	0	0	0	1	2	1	8	0.0
Integrating neuron										
RIA	19	(46)	4	1	2	1	17	7	14	0.11 ± 0.09
RIM	6	(21)	13	4	1	0	1	0	2	0.81 ± 0.17
RIB*	3	(6)	3	0	0	0	2	0	1	0.50 ± 0.41
Mock kill										
Wild type	17	(29)	23	3	0	0	0	0	3	0.90 ± 0.11
<i>ttx-1(p767)</i>	10	(20)	0	0	0	0	7	11	2	0.0
<i>ttx-3(ks5)</i>	15	(44)	0	0	0	0	42	0	2	0.0
<i>tax-6(p675)</i>	15	(34)	4	7	1	18	0	0	3	0.32 ± 0.16
<i>tax-2(p691)</i>	15	(25)	0	0	0	0	2	2	21	0.0
<i>tax-4(p678)</i>	10	(20)	0	0	0	0	0	3	17	0.0
Naive animals										
Wild type	110	(306)	216	42	2	2	27	6	11	0.84 ± 0.04
<i>unc-86(e1416)</i>	30	(56)	12	0	13	25	0	0	6	0.21 ± 0.11

Individual cells were killed in anaesthetized wild-type animals of L1 larval stage with a 440-nm laser microbeam using a VSL-337 Nitrogen-laser (Laser Science, Inc.), DLM-110 dye module (LSI), and Zeiss Axioskop microscope^{5,10}. After operation, each animal that had been grown at 20 °C to an adult stage on the NG plate seeded with bacteria as a food source¹¹ was assayed repeatedly (two to four times) on a radial thermal gradient. Each assay plate was evaluated using the phenotypic categories shown in Fig. 1, and all the evaluated phenotypes are shown. As a parameter to quantitatively assess thermotaxis, also shown are the fractions (p) of W(-) and C(+) phenotypes to the total number of assays (n), and their two standard deviations ($\pm 2\sqrt{p(1-p)/n}$). Cells were assigned based on their positions and morphologies^{5,12} and, when available, fluorescein dye-filling properties (ASK, ADL, ASI)¹³, chemosensory functions (ASE)^{5,14}, and the reactivity with anti-Unc-86 antibody (AIZ)⁹ were used as additional cues. Except for the case in which only one AFD cell on the right or left amphid sensillum was killed, a pair of indicated cell types were killed. When all amphid cells except AFD (ASE, ADF, ASG, ASH, ASI, ASJ, ASK, ADL, AWA, AWB and AWC) were killed, the animals of late L1 or early L2 larval stage were used for operation, because certain chemosensory functions are required during L1 stage for developmental decision to become either a dauer larva or an adult⁴. Of two animals which could reach adult stage after such an operation, one animal went through a dauer stage, but became an adult about a week later. Because the cell assignment was often uncertain for AIZ, which is known to migrate during L1 stage⁹, several cells that were expected to include AIZ were killed at the initial stage of the experiments. The AIY-killed animals were found to be slightly inactive on the assay plates (see text), but the overall length of tracks was sufficient to allow us to score their phenotypes. When AIYs were killed in combination with AFD or AIZ, the operated animals became particularly inactive, hardly moving on unseeded plates with a thermal gradient. RIB kills seemed to induce somewhat uncoordinated movement, as judged by tracks left on agar. Thus the effects of three different killings (marked by an asterisk) were not evaluated in detail. Of five animals in which AFD and AIZ neurons had been killed that exhibited the athermotactic phenotype (boxed), two avoided the coldest region of the assay plates (see Fig. 3 legend). To test the possibility that killing interneurons generally disrupts the coordinated regulation of motor neurons, thereby causing abnormal thermotaxis, we also killed AIM and RIC interneurons, which are unlikely to be closely associated with the neural circuit including AFD⁸. We did not find significant phenotypic abnormalities for the animals in which RIC and AIM were killed (data not shown). For mock kill controls, laser pulses were delivered on the agar pad near the anaesthetized animals, which were then recovered and treated in the same way as for laser-operated animals. Based on linear thermal gradient assays, *ttx-1(p767)* and *ttx-3(ks5)* mutants were cryophilic, *tax-6(p675)* mutants were thermophilic, and *tax-2(p691)* and *tax-4(p678)* mutants were athermotactic mutants² (I.M., unpublished results). Animals that received neither operation nor mock killing were used as 'naive' controls. The standard deviation for the fraction of W(+) and C(+) was derived by assuming independence among respective assays, the binomial distribution of normal (W(+) or C(+)) and abnormal (T(+), T(-), C(-), C/A(-) or A(-)) responses, and by the approximation of binomial to normal distribution. Because a relatively large fraction (14%) of naive wild-type animals showed the C(-) phenotype, we assumed that this phenotype does not represent abnormal thermotaxis and should be included in the normal responses. When applicable, the phenotypic spectrum of a particular kill was compared with that of naive wild-type animals by a chi-squared test using a 2 × 2 contingency table.

FIG. 1 Tracks of wild-type and laser-operated animals on a radial thermal gradient ranging from 17 °C to 25 °C. Responses of a wild-type animal cultivated at 15 °C (a), 20 °C (b) and 25 °C (c). Isothermal trackings observed at or near 15, 20 and 25 °C are thought to reflect the cultivation temperatures. d, The ttx phenotype of category W(+), in which the animal made clear isothermal circle(s) around 20 °C (a diameter of 4–5 cm) on the assay plate (b belongs to this category). e, The ttx phenotype of category C(+), in which the animal made clear isothermal circle(s) only around the coldest region, the central area (within a diameter of less than 3 cm) of the plate. f, The ttx phenotype of category C(–), in which the animal stayed in the coldest region for most of the time during the assay and did not leave clear isothermal tracks in any region. g, The ttx phenotype of category C/A(–) in which the animal visited or stayed in the central area for no more than half of the whole assay period, and no isothermal tracks were observed in any region. h, The ttx phenotype of category A(–), in which the animal either moved almost randomly or left significantly defective circular tracks on the assay plate. i, The ttx phenotype of category T(+), in which the animal almost never visited the central cold area, but stayed near the periphery for most of the time during the assay and left isothermal tracks in the region around or higher than 20 °C. j, The ttx phenotype of category T(–), in which is similar to T(+) except that no isothermal tracks were observed in any region. For clarity, tracks of animals were traced from assay plates representing the seven phenotypic categories in d–j, in which all the animals were cultivated at 20 °C for at least 4 h before the assay (see Table 1).

METHODS. A radial thermal gradient was established on agar medium



using a 9-cm plate and a vial containing frozen acetic acid, as described previously², except that each assay plate contained 8 ml of a medium consisting of 2% agar, 0.3% NaCl and 25 mM potassium phosphate buffer (pH 6.0). A single animal was placed on an assay plate, which had a thermal gradient ranging from ~17 °C in the central area to ~25 °C at the periphery, and allowed to move freely for 1.5 to 2.0 h. After the assay the animal was either killed or removed to reculture at 20 °C for the next assay, and tracks of the animal were photographed. Thermotactic phenotypes were evaluated based on these tracks.

characteristics are similar to those of certain thermophilic mutants such as *tax-6(p675)*² (Table 1; I.M. and Y.O., unpublished results). The phenotype of *unc-86* mutant animals supports the important role of AIZ in thermotaxis. Because of cell lineage abnormalities, the AIZ neurons in *unc-86* mutants are functionally abnormal⁹. In our assays, the animals carrying *unc-86(e1416)*, a putative null mutation by a variety of criteria (R. Baumeister, personal communication), indeed showed the responses characteristic of thermophilic mutants (Table 1).

To investigate the regulatory relationship between AFD, AIY and AIZ neurons, the effects of double killings were assessed (Table 1). When AIY neurons were killed together with AFDs or AIZs, the operated animals were found to be too inactive in assay plates for the phenotypes to be evaluated accurately. By contrast, the result of killing both AFD and AIZ neurons was informative. The phenotypes of all operated animals were evaluated as A(–), a phenotypic spectrum that is more abnormal than, and significantly different from, that of AFD-killed animals. This suggests that, although currently unknown, another thermosensory neuron (labelled X in Fig. 3b) independently activates AIZ.

The RIA interneuron could receive chemical input from both AIY and AIZ, and could be an important site for integrating various sensory informations⁸ (Fig. 2). The RIA-killed animals seemed partly defective in thermotaxis; animals lacking RIA displayed a wide phenotypic spectrum of abnormalities that does not resemble any other spectra shown (Table 1). The RIM motor neuron could also interact directly with both AIY and AIZ by gap junctions and chemical synapses, respectively⁸ (Fig. 2). Kill-

ing RIM, however, did not give rise to defective thermotactic responses (Table 1). The RIB interneuron could also be part of the circuit, because RIB is postsynaptic to AIY and presynaptic to AIZ and RIA⁸ (Fig. 2). When RIB neurons were killed, the operated animals seemed to become slightly uncoordinated on assay plates, although they were capable of moving isothermally (Table 1). As was the case when AFD and AIY neurons, and AIY and AIZ neurons, were killed, it was not possible to evaluate accurately the effects of killing RIB neurons.

As noted previously², migration to the cultivation temperature can be established conceptually by balancing two opposing drives, one for upward and the other for downward migration in a thermal gradient. It is possible that in some of the mistactic mutants, either cryophilic or thermophilic, one of these two drives is disrupted. We have demonstrated that eliminating the functions of AIY and AIZ mimicked the phenotypes of cryophilic and thermophilic mutants, respectively. Perhaps at the cellular level, AIY is responsible for upward drive and AIZ for downward drive; if so, counterbalancing the activities of these two neurons depending on the cultivation temperatures would be crucial for thermotaxis. How, at the cellular level, is the counterbalancing regulation by AIY and AIZ achieved? One plausible hypothesis is that these two neurons regulate one or more downstream neurons in opposite directions. RIA and RIM could directly receive chemical or electrical signals from both AIY and AIZ, and the phenotype of RIA-killed animals suggests that RIA is at least one such target (Fig. 2; Table 1). Further, the partial effect of killing RIA neurons suggests that other downstream neurons are indirectly regulated by AIY and AIZ.

FIG. 2 A neural network for amphid-related neurons constructed from the ultrastructural analysis (modified from ref. 8). Triangles, hexagons and a circle represent sensory neurons, interneurons and a motor neuron, respectively. Arrows indicate chemical synapses with their directions, H shapes indicate gap junctions, and U shapes indicate gap junctions between right and left of the same neuron type⁸. To reduce the complexity of the drawing, only connections between 20 neurons (12 sensory neurons, 7 interneurons and a single motor neuron) are shown, and some of their connections are indicated using neuron names.

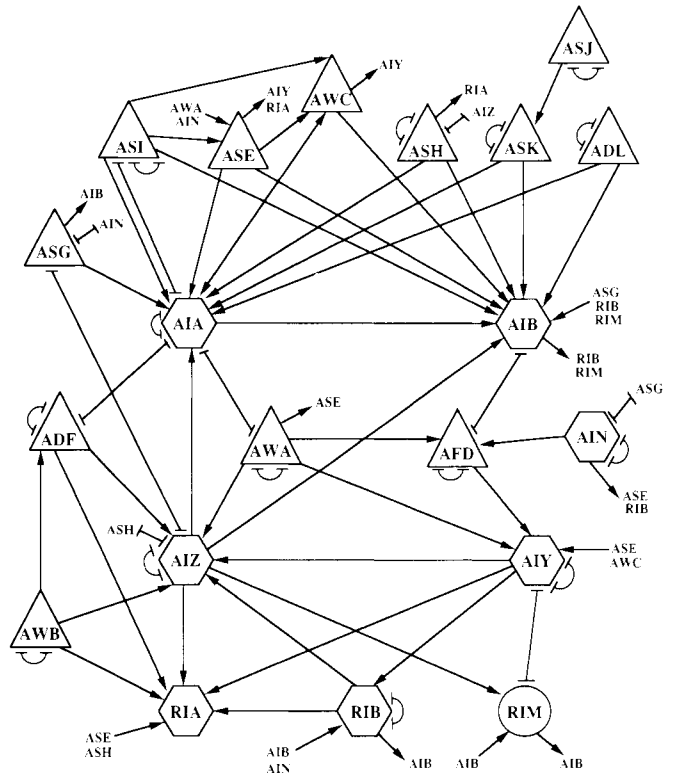
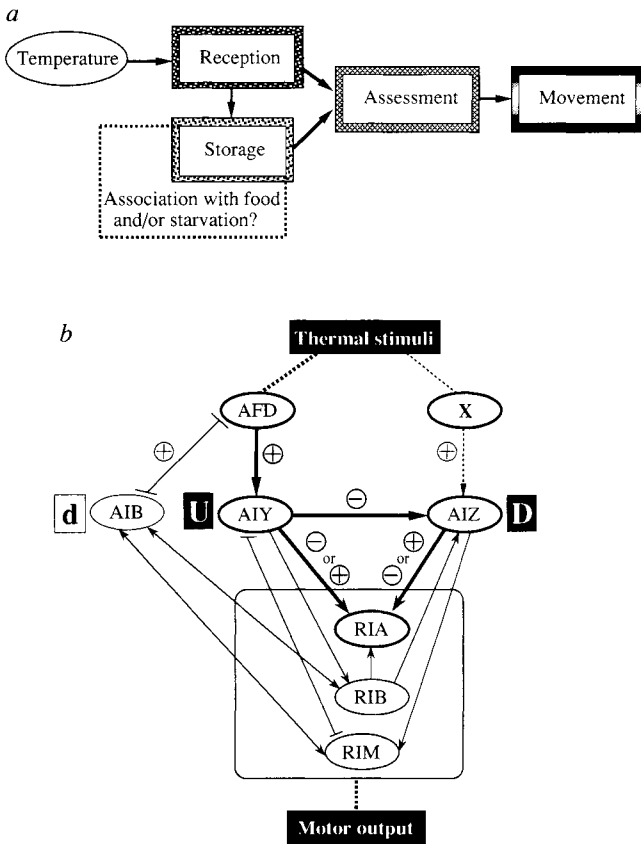


FIG. 3 Regulatory models of thermotaxis. *a*, A conceptual model. First, temperatures are sensed (Reception). Prolonged exposure (about 4 h) to the same temperature leads the animals to store that thermal information (Storage), possibly in association with food and/or starvation² (I.M. and Y.O., unpublished results). On a thermal gradient, the animals assess the changes in thermal information relative to the stored thermal information (Assessment). They thus migrate towards either colder or warmer regions, and move isothermally around the region at which the temperature coincides with the stored thermal information (Movement). *b*, A neural model proposed in the present study. Arrows indicate chemical synapses with their directions, and H shapes indicate gap junctions⁸ (see Fig. 2). An arrow with a thin dotted line indicates a neural pathway proposed in this work. A circled + sign indicates an activating signal, and a - sign indicates an inhibitory signal. A box represents a group of neurons including RIA, RIB, RIM and other inter- and motor neurons, which are possibly controlled by AIY, AIZ and AIB. AIZ is responsible for the major downward movement (movement to a lower temperature, D) and AIY for the major upward movement (movement to a higher temperature, U). Additionally, the phenotypic abnormality of AFD-killed animals, which was more severe than that of AIY-killed animals, can be explained if an additional interneuron target of AFD plays a minor and redundant role for the downward movement (Table 1). This interneuron is currently unknown, but AIB is a strong candidate for this unknown interneuron. Although the present study showed no direct evidence for the AIB function in thermotaxis (Table 1), it is reasonable to assume that the activation of AFD should activate AIB, because they are connected by gap junctions⁸ (Fig. 2). Thus we assigned the minor role for the downward movement to AIB (d) in this model. According to these assumptions, disturbing the d pathway only (AIB kills) has no significant effect, as long as both major D and U pathways are intact (Table 1). Activating the D pathway only (AFD kills) results in a phenotypic spectrum consisting of C(-), C/A(-) and A(-) (Table 1). Knocking out the U pathway and rescuing the D and d pathways (AIY kills) results in a strong cryophilic phenotype (C(-)) (Table 1). Knocking out the D pathway and rescuing the U and d pathways (AIZ kills) results in a thermophilic phenotype (T(+)) or T(-) (Table 1). Knocking out all pathways (AFD and AIZ kills) results in the most severe athermotactic phenotype (A(-)) and, at the same time, might induce or reveal some unknown thermosensory system with which the animals can sense and avoid cold temperatures (Table 1). Intriguingly, the d pathway through AIB could also be consistent with the results of AFD and AIY kills, and AIY and AIZ kills, if both of their phenotypic spectra were regarded similar to the spectrum resulting from AFD kills (Table 1).



through neurons that could receive direct inputs from either AIY or AIZ. Because AIY and AIZ neurons are heavily interconnected⁸, the counterbalancing regulation could also be achieved by direct interaction between AIY and AIZ.

Isothermal movement may be distinct in mechanism from migration to the cultivation temperature² (I.M. and Y.O., unpublished results). In the migration process the animals move along a thermal gradient of about $0.2\text{ }^{\circ}\text{C mm}^{-1}$ in a radial gradient assay, in which a relatively large temperature increase or decrease has to be assessed over time or in a locomotion-dependent fashion. In isothermal movement, the animals usually move perpendicularly to a thermal gradient, in which a small temperature change ($<0.1\text{ }^{\circ}\text{C}$) has to be assessed without changing the overall direction. Because isothermal movement allows the animals to stay around the cultivation temperature, we propose that isothermal movement reflects a process of expressing a preference for a temperature. Thus our observation that killing either AFD or AIY abolished isothermal movement suggests that the mechanism for thermal preference expression involves the neural circuit including AFD and AIY but not AIZ.

Our results are consistent with the regulatory model summarized in Fig. 3b. Thermal stimuli are sensed by AFD, and possibly by a neuron X ('Reception' in Fig. 3a). AFD activates AIY and possibly AIB, and the neuron X activates AIZ (see Fig. 3 legend for the involvement of AIB in the pathway). AIY and AIZ could establish the counterbalancing regulation in two ways ('Assess-

ment' and/or 'Movement' in Fig. 3a). AIY could regulate AIZ directly by inhibiting AIZ by its chemical synapses, or indirectly by inhibiting (activating) downstream neurons such as RIA while AIZ activates (inhibits) them. This model provides testable hypotheses for the cellular and molecular bases of thermosensation and thermal adaptation. □

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1. Eckert, R., Randall, D. & Augustine, G. (eds) *Animal Physiology* (Freeman, New York, 1988).
2. Hedgecock, E. M. & Russell, R. L. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4061–4065 (1975).
3. Perkins, L. A., Hedgecock, E. M., Thomson, J. N. & Culotti, J. G. *Dev. Biol.* **117**, 456–487 (1986).
4. Bargmann, C. I. & Horvitz, H. R. *Science* **251**, 1243–1246 (1991).
5. Bargmann, C. I. & Horvitz, H. R. *Neuron* **7**, 729–742 (1991).
6. Bargmann, C. I., Hartwig, E. & Horvitz, H. R. *Cell* **74**, 515–527 (1993).
7. Kaplan, J. M. & Horvitz, H. R. *Proc. natn. Acad. Sci. U.S.A.* **90**, 2227–2231 (1993).
8. White, J. G., Southgate, E., Thomson, J. N. & Brenner, S. (eds) *Phil. Trans. R. Soc.* **B314**, 1–340 (1986).
9. Finney, M. & Ruvkun, G. *Cell* **63**, 895–905 (1990).
10. Avery, L. & Horvitz, H. R. *Cell* **51**, 1071–1078 (1987).
11. Brenner, S. *Genetics* **77**, 71–94 (1974).
12. Sulston, J., Schierenberg, E., White, J. G. & Thomson, J. N. *Dev. Biol.* **100**, 64–119 (1983).
13. Hedgecock, E. M., Culotti, J. G., Thomson, J. N. & Perkins, L. A. *Dev. Biol.* **111**, 158–170 (1985).
14. Ward, S. *Proc. natn. Acad. Sci. U.S.A.* **70**, 817–821 (1973).

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Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP

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THE Rubinstein-Taybi syndrome (RTS) is a well-defined syndrome with facial abnormalities, broad thumbs, broad big toes and mental retardation as the main clinical features^{1–3}. Many patients with RTS have been shown to have breakpoints in, and microdeletions of, chromosome 16p13.3 (refs 4–8). Here we report that all these breakpoints are restricted to a region that contains the gene for the human CREB binding protein (CBP), a nuclear protein participating as a co-activator in cyclic-AMP-regulated gene expression^{9–12}. We show that RTS results not only from gross chromosomal rearrangements of chromosome 16p, but also from

point mutations in the CBP gene itself. Because the patients are heterozygous for the mutations, we propose that the loss of one functional copy of the CBP gene underlies the developmental abnormalities in RTS and possibly the propensity for malignancy.

The chromosomal region around the RTS translocation breakpoints and microdeletions was cloned by isolating a set of overlapping cosmids. We were able to situate all RTS breakpoints in an area of 150 kilobases (kb) using fluorescence *in situ* hybridization (FISH) (Fig. 1a). The microdeletions were found to range between 130 and >650 kb (Fig. 1b). To find genes in the candidate region a single-copy subclone from cosmid RT1 was used to screen a human fetal brain complementary DNA library. One positive cDNA (cRTO), containing a poly(A) tail and directed towards the RTS breakpoints, was used to identify adjacent 3' sequences. 5' cDNA clones were obtained by screening a human Graves' disease thyroid cDNA library with the cDNA previously described⁹. All of the cDNAs identified in this search show very high (85–90%) sequence homology with the murine CBP⁹, a protein that participates in cAMP-regulated gene expression. FISH was used to show that murine CBP (mCBP) hybridizes to human chromosome 16p13.3 (not shown). Further mapping studies indicate that the most 5' mCBP fragment (bp 1–421) hybridizes to cosmid RT166 and the most 3' fragment (bp 5,600–7,326) to RT1. Thus the human CBP gene spans at least the 150-kb genomic area containing the RTS breakpoints (Fig. 1a).

Disruption of the CBP gene in RTS translocations was analysed by Southern and northern hybridization. We found cases with aberrant genomic fragments (Fig. 2a), as well as truncated messenger RNA (Fig. 2b). To search for mutations in RTS patients without rearrangements, we used the protein truncation test (PTT)¹³. In 2 out of 16 patients examined, a truncated protein product was found (Fig. 3). Sequence analysis confirmed these results (Fig. 4). In both patients, who had classical RTS phenotypes, a codon for glutamine was changed into a stop codon by a C→T substitution. In patient 9 the mutation destroyed a *PvuII* restriction site. This site was found to be present in both chromosomes of the parents, indicating a *de novo* mutation (not shown).

The microdeletions, translocations and point mutations all implicate CBP derangement as cause of RTS. It cannot be excluded that the region may contain more coding sequences. We detected five HTF islands in the 850-kb cloned region. Such islands are often associated with 5' ends of genes¹⁴. Only one HTF island is located in the area of interest (cosmid RT166) and could correspond to the 5' end of CBP. The other four HTF

islands map around the more centromeric N2 marker, which is not deleted in RTS patients (Fig. 1b).

The occurrence of point mutations in a single gene implies that RTS is not caused by a contiguous-gene syndrome, in which the deletion of several adjacent genes causes a composite set of abnormalities, as has been thought so far. We explain the relation between a single mutated gene and the broad range of

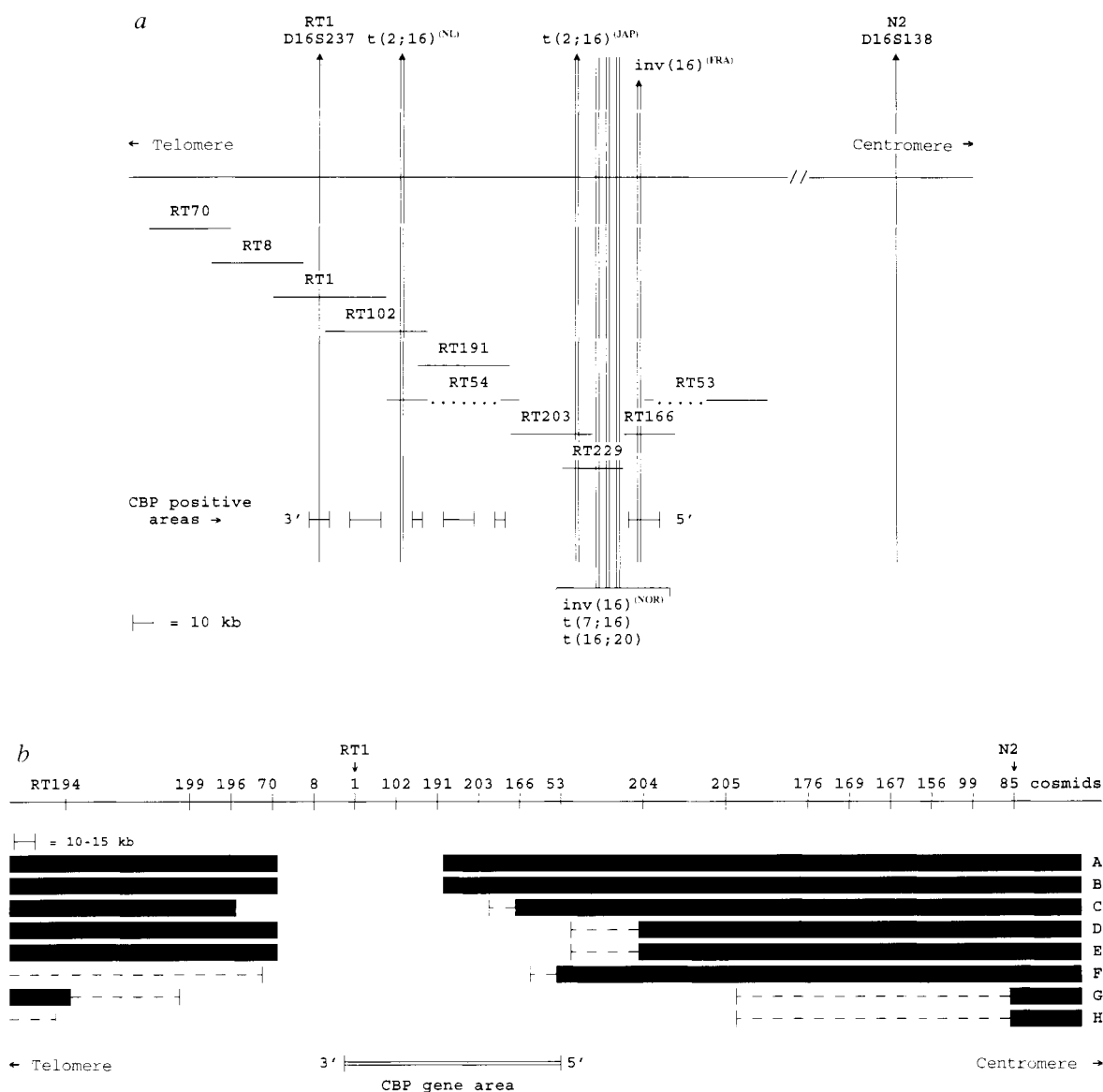


FIG. 1 A section of the ~850-kb cosmid contiguity in the RT1-N2 area. The RTS $t(2;16)^{NL}$ breakpoint is mapped by Southern analysis to a 5.2-kb *EcoRI* fragment of cosmid RT102, the $inv(16)^{FRA}$ to a 9-kb *BamHI* end fragment of RT166 and the $t(2;16)^{JAP}$ to a 4-kb *AatII* end fragment of RT203 (not shown). We used cosmids from the area as probes on metaphase spreads in a FISH²⁶ analysis to establish that the three other RTS breakpoints map in cosmid RT229 (not shown). Solid lines represent cosmids. Dotted lines represent the deletions in the yeast artificial chromosome (YAC) subclones RT53 and RT54. b, Schematic representation of 8 RTS microdeletions. Black bars represent the undeleted DNA. Broken lines represent uncloned or untested areas. The smallest deletions are ~130 kb in size (patients A and B); the largest is >650 kb (patient H). Microdeletions G and H extend >200 kb beyond the apparent 5' end of CBP, therefore probably removing the entire gene.

METHODS. a, Cosmids CN23 (=N2) and RT1 were used as starting points for cosmid walking in a chromosome-16-specific library (Los

Alamos). A *PstI* single-copy subclone was isolated from RT1, which showed conservation with DNA from several species. PCR primers were chosen from this subclone for screening the St Louis YAC library²⁷. One of them, 262A3, was subcloned into cosmids, of which only RT53 and RT54 are depicted. Sizes of the remaining gaps in the ~850-kb contiguity were estimated by fibre FISH with the use of the DIRVISH protocol²⁸. The DIRVISH results, as well as restriction-map analysis of the cosmids in the area, indicate that the 440-kb YAC contains deletions. For this reason, we constructed our contiguity based on cosmids from the chromosome-16-specific library and not on YAC subclones. b, Metaphase FISH²⁶ was done on the depicted cosmids from the region. For every cosmid, 30 metaphases per patient were examined to determine whether one or both chromosomes 16 showed fluorescent dots. At least 27 of the 30 must lack cosmid signal to be scored as deleted. The chromosomes 16 were identified by their sizes and by the bright diaminophenylindole staining of their heterochromatic regions.

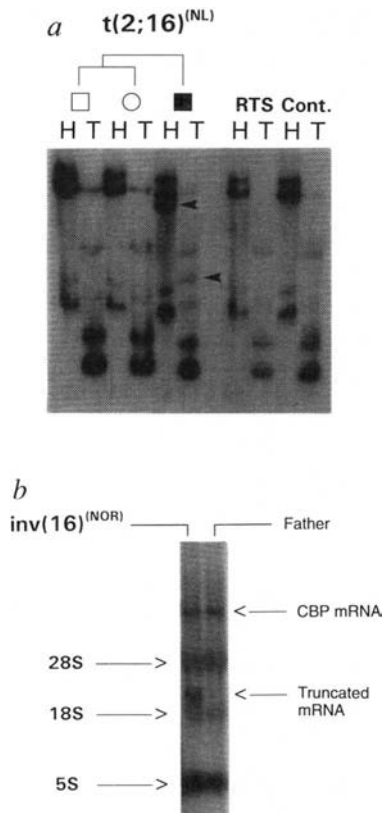


FIG. 2 *a*, Translocation disrupting the CBP gene in a Dutch RTS patient. Southern blot of *Hind*III (H) and *Taq*I (T) digested DNA of a RTS patient with a *de novo* t(2;16). From left to right, samples from: the father, the mother, the patient, an RTS patient without rearrangement or RT1 microdeletion, and a control. A fragment of the mCBP was used as a probe. One aberrant band can be seen with each enzyme (indicated by arrowheads) in the t(2;16) patient's lanes but not in the lanes of parents and controls. Two aberrant bands are expected to hybridize to a probe spanning the breakpoint. The absence of the second band could be due to the small size of the second band or by a concomitant deletion. *b*, Aberrant CBP transcript in a patient with RTS. Northern blot of total RNA isolated from an Epstein-Barr-virus (EBV)-transformed lymphoblastoid cell line of a Norwegian RTS patient with a *de novo* inv(16) and his father. As probe a 5' murine CBP fragment (base pairs 1–421) was used.

METHODS. *a*, Southern analysis was done with a mixture of the mCBP fragments 2,160–3,294 and 3,294–5,032 as a probe. These two fragments hybridize to the proximal and distal genomic *Eco*RI fragments of this translocation, respectively. Combining these fragments in one hybridization results in a cDNA probe that spans the t(2;16)^(NL) breakpoint. Hybridization was done at 65 °C under competition with an excess of total human sonicated placental DNA to suppress hybridization of repeated sequences. The blot was washed with a stringency of 0.3 × SSC/0.1% SDS. *b*, Hybridization was done at 41 °C for two days and the blot washed with a stringency of 1 × SSPE. We used the mCBP fragment 1–421 as a probe. The positive signal with 28S, 18S and 5S ribosomal RNA may be due to the small probe size. A larger fragment of CBP was not used because of the high homology with p300^{15–17}.

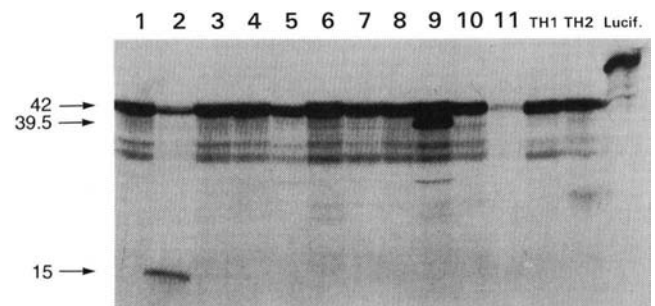
symptoms as seen in RTS by the role of CBP as transcriptional co-activator. CBP mediates cAMP-regulated gene expression, by interacting with the phosphorylated form of CREB^{9–12}, a transcription factor that binds to a DNA element known as the cAMP-regulated enhancer (CRE). Therefore an altered CBP can be predicted to influence a large set of target genes of CREB or other transcription factors that interact with CBP.

In both cases resulting from CBP point mutations the CREB binding site (residues 462–661)⁹ is lost by the truncation.

These truncated proteins would not be predicted to interact with CREB. Several of the microdeletions also remove the CREB binding site or the entire CBP molecule (Fig. 1*b*). The existence of both of these types of mutations makes a dominant negative model unlikely. A haplo-insufficiency model, in which two functional copies of the gene are required to produce sufficient product for normal development, seems to be a more likely explanation for the RTS phenotype. CBP is highly related to p300^{15–17}, a nuclear target of the adenovirus

FIG. 3 Truncated CBP proteins in two patients with RTS. The protein truncation test (PTT), a mutation detection system based on *in vitro* transcription and translation, performed on a 5' 1.1-kb CBP polymerase chain reaction (PCR) product of non-rearrangement RTS patients. The 1.1-kb PCR product corresponds to a 42K protein. Samples from patients 2 and 9 show a calculated truncated protein of 15K and 39.5K, respectively. Results from 11 patients are depicted on this autoradiogram. Lanes marked TH are the control individuals, whereas the luciferase lane contains a control for the translation reaction. Samples of 5 additional patients were analysed on another gel but did not reveal truncated proteins. Markers for relative molecular mass (K) are shown alongside.

METHODS. Total RNA was isolated from EBV-transformed lymphoblastoid cell lines of the patients and reverse transcribed (RT) with random hexamers. A pool of total human cDNA was produced in this way. Expression levels of CBP proved to be high enough to perform a one-step RT-PCR, directly using the PTT-primers (that is, a eukaryotic T7-promoter sequence added to a 5' gene-specific sequence and a second reverse-orientated gene-specific primer) on the cDNA pools. Primer pRT7 (5'-GGATCCTAATACGACTCACTATAGGCGCGAGCAGGTGAAAATGG-3') and pRT6 (5'-GCGAGCAGGCCCGAACCTCTC-3') were used as PTT primers. PTT often requires the use of a primary PCR on which a second PCR is performed using the PTT primers in a (secondary) nested or semi-nested PCR reaction. In lane TH2 the PTT primers were used in such a semi-nested fashion. In general, a eukaryotic in-frame translation initiation sequence is included in the 5' PTT-primer. Here we used the endogenous translation initiation site, resulting in a PCR product containing 15 bp of the 5' untranslated region and 1,144 bp of the transcribed sequence. Further PTT protocol was performed as described elsewhere¹³.



Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice

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CD19 is the hallmark differentiation antigen of the B lineage. Its early expression has implicated a role for CD19 during the antigen-independent phases of B-cell development, whereas in mature B cells CD19 can act synergistically with surface immunoglobulin to induce activation¹. We have generated CD19-deficient mice and found that development of conventional B cells is unperturbed. However, mature *CD19*^{-/-} B cells show a profound deficiency in responding to protein antigens that require T-cell help. This is accompanied by a lack of germinal centre formation and affinity maturation of serum antibodies. Thus CD19 is crucial for both initial B-cell activation by T-cell-dependent antigens and the maturation and/or selection of the activated cells into the memory compartment. An impairment in ligand-driven selection may also be responsible for the observation of a striking reduction in the B-1 (formerly Ly-1) B-cell subset, thought to develop under the control of self-antigens and bacterial antigens (reviewed in ref. 2).

CD19-deficient mice were obtained by inactivation of the gene in embryonic stem cells (Fig. 1) and subsequent germline transmission of the mutant allele through the generation and breeding of chimaeric mice. Despite the early expression and signalling ability of CD19 (reviewed in refs 3, 4), analysis of bone-marrow cells from homozygous mutant mice revealed normal development and expansion of B lineage cells, as indicated by the number and distribution of pro-B (IgM⁺B220^{lo}CD43⁺), pre-B (IgM⁺B220⁺CD43⁺), immature B (IgM⁺B220⁺CD43⁺) and resting mature (IgM⁺B220^{hi}CD43⁺) IgD-expressing B cells (Fig. 2). Analysis of spleen (Fig. 2), Peyer's patch and lymph node from *CD19*^{-/-} mice did not reveal differences in the number or surface phenotype of conventional B cells.

B-1 cells, which predominate in the peritoneal cavity, account for most serum IgM and are distinguished from conventional (B-2) B cells in surface phenotype, antigen specificity, signalling, and growth properties (reviewed in ref. 2). In contrast to B-2 cells, we find B-1 (CD23⁺IgM^{hi}IgD^{lo}) cell formation to be severely affected by the loss of CD19 (Fig. 2). This is underscored by a reduction in serum IgM (60–90 compared with 250–300 µg ml⁻¹), and in the percentage of peritoneal lymphocytes bearing the B-1-restricted specificity for the self-membrane phospholipid, phosphatidyl choline⁵ (0.2–0.5% compared with 12–15% of IgM-positive lymphocytes). In that the decrease in the B-1 cell number (10–20% of controls) persists, even in mice of several months of age, we speculate that CD19 is required for optimal antigen-driven expansion and maintenance of this population.

CD19 on mature B cells is associated with TAPA-1 (CD81), Leu 13 and complement receptor 2 (CD21)^{6,7} which act synergistically with surface immunoglobulin to promote B-cell activation^{1,8,9}. To assess B-cell function in the absence of CD19, mice were immunized with T-cell-independent type 2 (TI-2) and T-cell-dependent (TD) antigens. The hapten-specific response to the TI-2 antigen 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll, known to be derived from B-2 cells¹⁰, was comparable to that

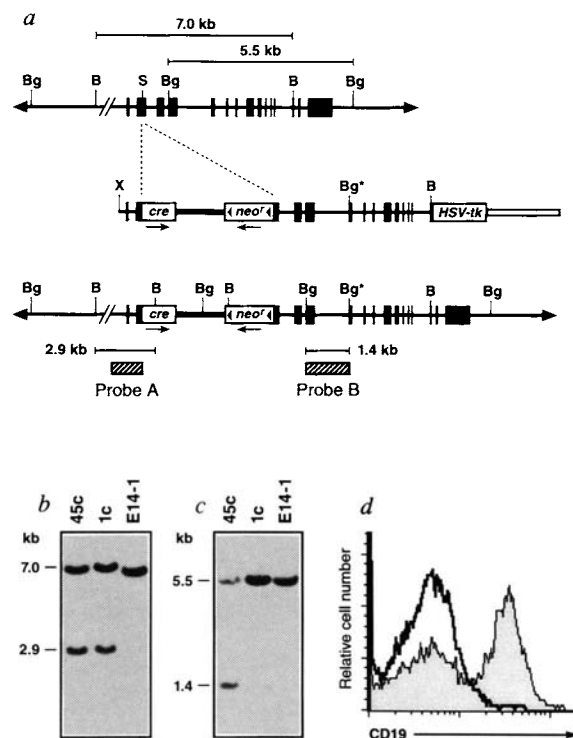


FIG. 1 a, Inactivation of the CD19 gene in ES cells. Configuration of the locus before²⁴ (top) and after (bottom) homologous recombination with the targeting vector (middle). Exons (black boxes), lengths of diagnostic restriction fragments, and location of probes are shown. The coding sequence was disrupted by insertion of a cassette that encodes the P1-bacteriophage-derived Cre recombinase followed by a rabbit β-globin intron sequence and polyadenylation signal, and a neomycin resistance gene flanked by FRT sequences (triangles). The HSV-tk gene was appended to allow for selection against random integration. These features allow selection of ES cell transfectants and subsequent deletion of the resistance marker in homologous recombinants using the FLP/FRT recombination system²⁵. This approach leads to the functional inactivation of CD19, while providing B-cell-specific expression of the *cre* gene, further allowing germline genes conditioned for Cre-mediated deletion²⁶ to be deleted only in B cells (unpublished results). B, BamHI; Bg, BglII; S, SmaI; X, XhoI. b, c, Southern blot analysis. b, Homologous recombinants identified by polymerase chain reaction (PCR) (no. 45c and no. 1c used to derive *CD19*^{-/-} lines are shown) displayed the restriction patterns predicted for a homologous recombination event (2.9-kilobase (kb) BamHI fragment, probe A). c, One of the clones (no. 45c) carried the frame shift mutation in the transmembrane exon (1.4-kb BglII fragment, probe B). d, *CD19*^{-/-} F₂ mice were identified by Southern blot and staining of peripheral blood lymphocytes with an anti-CD19 antibody (ID3). Mice derived from clones 1c and 45c were indistinguishable in phenotype.

METHODS. The targeting vector (a, middle) was constructed by PCR-amplification of ES (E14-1)-cell DNA using oligonucleotides tagged with restriction sites at the 5' end. Primers were designed to (1) amplify a 6.8-kb genomic fragment spanning from 330 bp upstream of exon 1 to exon 13, (2) introduce a frame shift mutation into the transmembrane exon (BglII site in exon 5 (Bg*)), (3) disrupt the CD19 translation initiation consensus sequence and provide a cloning site for insertion of the *cre/neo* cassette (XhoI/SalI fragment). The following primers were used²⁴: 5' exon 1, 5'-TCTAAGCTTAATATTTAACAGGTGCCATATGTGA/TAGATGCATCCCTGGGGCACCCTGCTCTGCTG-3'; exons 1–5, 5'-CAG-ATGCATCTGGCTCCCGTGCCATCTCTCTCCCTGCTCTCC/TCCAGATCTCCAG-TTCTCAACAGCCAGAGCCACAC-3'; exons 5–13, 5'-ACTAGATCTGGA-TGGATAGTCCAGTGGTGACTTTAG/TGCATCGATGGATCCCTCATATCTT-CATAGGAC-3'. ES cell culture and generation of chimaeric mice were carried out as described²⁷. For transfection, the targeting vector was linearized with XhoI (introduced by linker addition to the BspI restriction site 270 bp 5' of exon 1). PCR-positive G418^r clones (results not shown) were confirmed by Southern blot (b, c).

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of normal mice over a wide dose range (Fig. 3A). In the response to the TI-2 antigen $\alpha(1-3)$ -dextran, $CD19^{-/-}$ mice produced fivefold less serum antibody (results not shown); however, B-1 cells are known to contribute substantially to this response¹⁰. Thus, taking the diminished B-1 cell compartment into account, it appears that CD19 is not required for B-cell activation by TI-2 antigens.

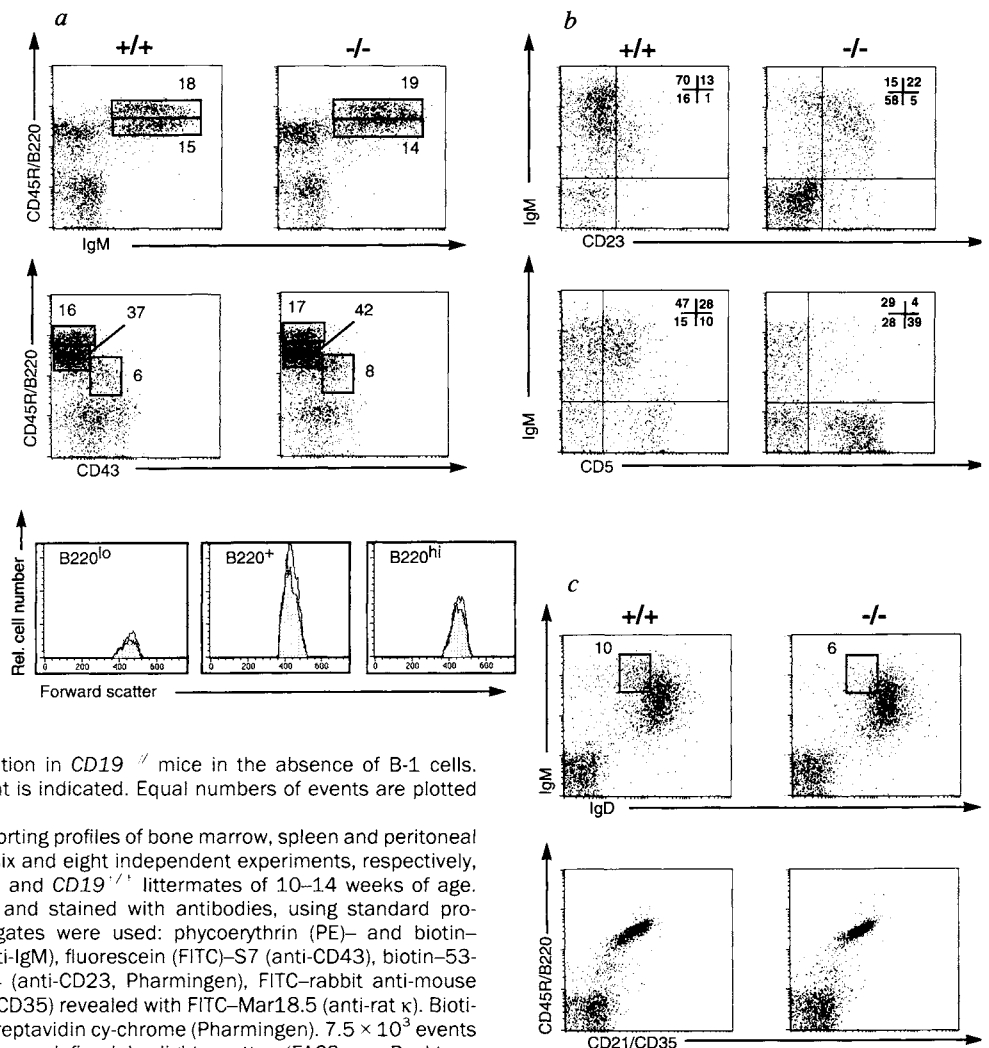
CD19-deficient B cells are severely impaired in their responses to TD antigens, a condition initially suggested by lower levels of serum IgG1 and IgE than in control mice ($0.7-15$ compared with $150-300 \mu\text{g ml}^{-1}$ and $\leq 0.1-6.0$ compared with $30-100 \mu\text{g ml}^{-1}$, respectively). This deficiency is revealed by the substantially decreased hapten-specific antibody response by an NP-chicken- γ -globulin conjugate (NP-CG) (Fig. 3B). NP-specific B cells recruited into this response normally undergo affinity maturation in the germinal centre (GC) through a process of somatic mutation to give rise to plasmablasts or memory cells expressing high-affinity λ_1 -bearing IgG1 antibodies^{11,14}. We find that $CD19^{-/-}$ mice respond to NP-CG with similar kinetics to control mice, but the magnitude of the response is reduced 10- to 100-fold (Fig. 3B). Moreover, there is a virtual absence of high-affinity NP-specific IgG1 antibody (Fig. 3C), accompanied by a lack of GC formation (Fig. 3D). These data strongly suggest that antigen-specific $CD19^{-/-}$ B cells are not activated efficiently in the periarteriolar lymphocyte sheath, resulting in poor primary responses, and those migrating to the follicles are not selected efficiently to generate GC and memory B cells.

The compromised TD responses could reflect inadequate T-cell priming or decreased B-cell responsiveness. The latter does not seem to be so, in that *in vitro* culture of $CD19^{-/-}$ B cells revealed no impairment in the upregulation of major histocompatibility complex (MHC) class II, CD23 (results not shown), B7-1 (CD80; results not shown) and B7-2 (CD86) molecules (Fig. 4a), nor in blast formation and proliferation in response to lipopolysaccharide (LPS), IL-4, CD40L and soluble (results not shown) or immobilized anti-IgM stimulation (Fig. 4b). To evaluate early signal-transduction events, we measured Ca^{2+} mobilization and protein tyrosine phosphorylation. $CD19^{-/-}$ splenic B cells were found to respond to surface-immunoglobulin crosslinking by increasing intracellular $[\text{Ca}^{2+}]$ (results not shown). Surface-immunoglobulin stimulation also induced tyrosine phosphorylation; however, we could not readily distinguish differences between CD19-deficient and normal B cells in either the pattern or the degree of phosphorylated substrates (results not shown).

To examine the ability of $CD19^{-/-}$ B cells to co-stimulate T cells, purified CD4^+ T cells and LPS-blast B cells were co-cultured in the presence of immobilized anti-CD3 antibody¹⁵. No significant difference in T-cell proliferation was detected between $CD19^{-/-}$ and syngeneic 129/Sv mice (Fig. 4c). Likewise, LPS-blast 129/Sv- and $CD19^{-/-}$ B cells were equally capable of stimulating proliferation of allogeneic BALB/c T cells (results not shown). Collectively, these initial findings do not support a direct co-stimulatory activity for CD19 acting through a putative coun-

FIG. 2 Representative flow cytometric analyses of bone marrow, peritoneal cavity and spleen from CD19 homozygous mutant ($-/-$) and normal ($+/+$) littermates. **a**, Bone marrow. Populations of pro-B ($\text{IgM}^{\text{B220}^{\text{lo}}}\text{CD43}^+$), pre-B ($\text{IgM}^{\text{B220}^{\text{lo}}}\text{CD43}^-$), immature B ($\text{IgM}^{\text{B220}^{\text{hi}}}\text{CD43}^-$) and mature B cells ($\text{IgM}^{\text{B220}^{\text{hi}}}\text{CD43}^+$) in the bone marrow are framed and percentages noted. Histogram below shows forward light scatter properties of gated B220^+ cells; $CD19^{+/+}$ (shaded), $CD19^{-/-}$ (solid line). The total number of nucleated cells recovered from the bone marrow of $CD19^{-/-}$ and $CD19^{+/+}$ mice was $(1.9 \pm 0.3) \times 10^7$ and $(1.8 \pm 0.4) \times 10^7$, respectively. **b**, Spleen. The lower percentage of splenic $\text{IgM}^{\text{hi}}\text{IgD}^{\text{lo}}$ cells (framed) in $CD19^{-/-}$ mice is attributed to fewer B-1 cells, as the $\text{CD23}^{\text{hi}}\text{IgM}^{\text{hi}}$ splenic population was found to be sharply reduced in CD43^+ cells²⁸ (results not shown). Expression of CD21/CD35, CD22 (results not shown) and MHC class II (results not shown) was found to be normal. **c**, Peritoneal cavity. B-1 cells decreased by 80–90%, including the CD5^+ subset. T cells are $\text{CD5}^{\text{hi}}\text{IgM}^{\text{B220}^-}$ and represent the dominant peritoneal lymphocyte population in $CD19^{-/-}$ mice in the absence of B-1 cells. The percentage of cells in each quadrant is indicated. Equal numbers of events are plotted for each staining.

METHODS. Fluorescence-activated cell sorting profiles of bone marrow, spleen and peritoneal cavity cells are representative of three, six and eight independent experiments, respectively, conducted with groups of four $CD19^{-/-}$ and $CD19^{+/+}$ littermates of 10–14 weeks of age. Single-cell suspensions were prepared and stained with antibodies, using standard procedures. The following antibody conjugates were used: phycoerythrin (PE)– and biotin–RA36B2 (anti-B220), PE–R33-24-12 (anti-IgM), fluorescein (FITC)–S7 (anti-CD43), biotin–53-7.3 (anti-CD5, Pharmingen), FITC–B3B4 (anti-CD23, Pharmingen), FITC–rabbit anti-mouse IgD antiserum (Nordic), 7G6 (anti-CD21/CD35) revealed with FITC–Mar18.5 (anti-rat κ). Biotinylated antibodies were detected with streptavidin cy-chrome (Pharmingen). 7.5×10^3 events were analysed with a lymphocyte gate as defined by light scatter (FACScan, Beckton–Dickinson).



ter-receptor on the responding T cell. We are currently investigating whether $CD19^{-/-}$ B cells respond normally to activated T cells *in vitro*.

The most striking parallel with the CD19 defect derives from the observation of profound deficiencies in antibody responses of humans and animals with complement (C3) deficiencies (reviewed in ref. 16), and from *in vivo* blocking studies with soluble CD21 or anti-CD21 antibodies¹⁷⁻¹⁹. Binding of C3 fragments by CD21 primes B cells for activation through surface

immunoglobulin²⁰⁻²². This synergism is likely to be mediated by CD19 and may occur *in vivo* by the co-recognition of opsonized foreign antigen or immune complexes by surface immunoglobulin and the CD19/CD21 complex²³. The normal response of $CD19^{-/-}$ mice to the TI-2 antigen NP-Ficoll suggests that antigens capable of inducing a high degree of surface-immunoglobulin crosslinking may override a CD19 dependence. In contrast, CD19 appears to be critical in mediating an efficient antibody response to TD antigens of limited crosslinking capac-

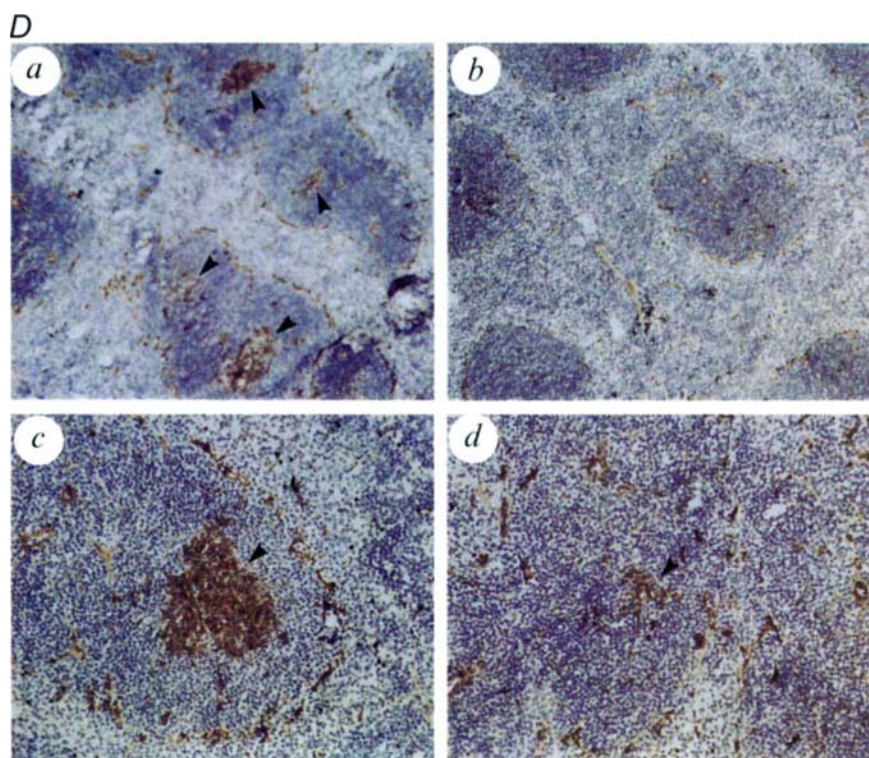
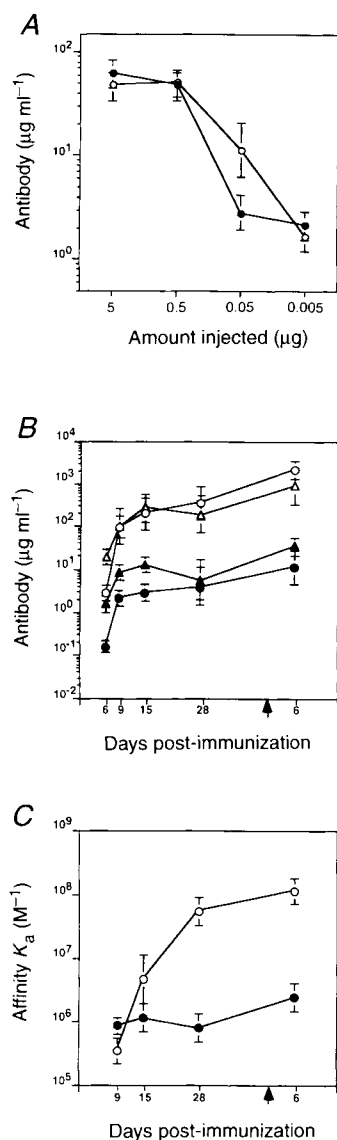


FIG. 3 Responses of $CD19^{-/-}$ (filled symbols) and normal (open symbols) littermates to TD and TI-2 antigens. A, Titres of λ -bearing NP-specific serum antibody at day 7 after immunization with various doses of NP₂₇-Ficoll are shown and are also representative of IgG3 and IgM NP-specific antibodies. B, Production of λ -bearing (triangles) and IgG1 (circles) NP-specific serum antibody at various time points following primary and secondary immunization with 5 μ g NP₂₀-CG. Immunization with 100 μ g of NP₂₀-CG elicited similar titres of λ -bearing (7.0 ± 0.5 compared with $\leq 0.2 \mu$ g ml⁻¹ at day 5, and 480 ± 80 compared with $20 \pm 0.4 \mu$ g ml⁻¹ at day 15 in $+/+$ and $-/-$ mice, respectively) and IgG1 (300 ± 40 compared with $5 \pm 2 \mu$ g ml⁻¹ at day 15 in $+/+$ and $-/-$ mice, respectively) NP-specific serum antibody. C, Affinity maturation of NP-specific IgG1 serum antibody. Arrows in B and C indicate the time-point of secondary immunization. D, Lack of GC in $CD19^{-/-}$ mice. Sections of spleen obtained at day 10 after immunization were stained with peanut agglutinin (PNA) (brown) and counterstained with haematoxylin (blue). Germinal centres (PNA⁺; arrows) were present at a frequency of

10–15 per section in normal littermates (a, c), but were not observed in $CD19^{-/-}$ mice (b). d, Small intrafollicular clusters of PNA⁺ cells were found at a frequency of <1 per section in $CD19^{-/-}$ mice but, in contrast to normal littermates, were not recognizable in serial sections. Magnifications: a, b, $\times 40$; c, d, $\times 100$.

METHODS. Mice (Igh^a/Igh^b allotype) of 10–14 weeks of age were immunized intraperitoneally with alum-precipitated NP-CG (primary and secondary), or the indicated amount of NP₂₇-Ficoll in PBS. Geometric mean values $\pm$ s.e.m. obtained from 3 or 4 mice per group are shown. Enzyme-linked immunosorbent assays were done as previously described for NP-conjugated antigens²⁹. Affinity maturation of serum IgG1 antibody was assessed by measuring the ratio of NP₄-BSA/NP₁₄-BSA (ref. 29), which is proportional to the binding constant (K_a). Splenic sections (6 μ m) were stained with PNA-biotin, detected with streptavidin-horseradish peroxidase, developed with diaminobenzidine, and counterstained with haematoxylin by standard procedure.

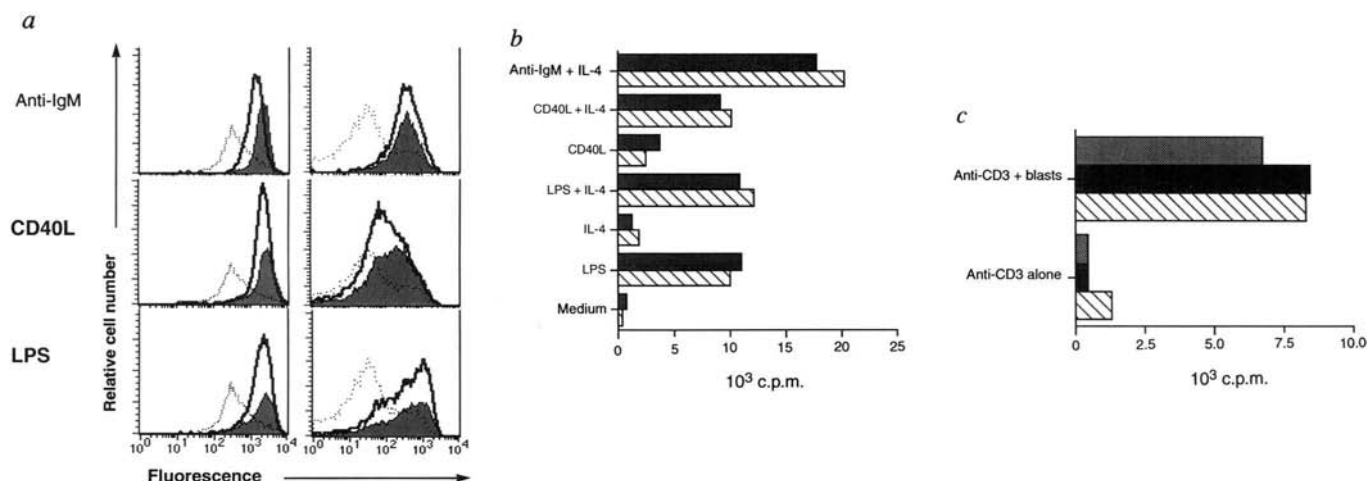


FIG. 4 *In vitro* stimulation and proliferation of splenic T and B cells from $CD19^{-/-}$ and normal mice. **a**, Expression of MHC class II (left column) and B7-2 (right column) by splenic B cells from $CD19^{-/-}$ (shaded) and normal littermates (solid line) after culture for 48 h in the presence of recombinant CD40L, LPS or anti-IgM plus IL-4. Staining of unstimulated cells is indicated by a dotted line. **b**, Proliferation of splenic B cells from $CD19^{-/-}$ (hatched bar) and normal littermates (filled bar) in response to recombinant CD40L, IL-4, LPS or anti-IgM. **c**, Proliferation of $CD4^{+}$ T cells in response to anti-CD3 antibody alone, or co-stimulation with syngeneic LPS-activated B cells. 129/Sv T cells and B cells (grey bar), 129/Sv T cells and $CD19^{-/-}$ B cells (filled bar), $CD19^{-/-}$ T cells and 129/Sv B cells (hatched bar).

METHODS. Splenic B cells were prepared by depletion of T cells and macrophages with anti-CD5 and anti-Mac1 coated magnetic beads (Miltenyi Biotec, Cologne). B cells (2×10^5 per well) were cultured in

complete RPMI medium supplemented with 50 ml l^{-1} anti-IgM (R33-24-12) coupled to Sepharose beads; 200 ml l^{-1} recombinant CD40L; $20 \mu\text{g ml}^{-1}$ LPS; or 5 ng ml^{-1} IL-4 (Genzyme). Expression of MHC class II and B7-2 was detected by surface staining with FITC-M5/114 (anti-IgA) and FITC-GL1 antibodies, respectively. The co-stimulation assay was performed as described previously¹⁵ using 5×10^4 sorted (FACStar PLUS, Beckton Dickinson) $CD4^{+}$ (GK1.5-PE antibody) T cells and 1×10^5 mitomycin C (Sigma) treated B cells. Wells were coated with $10 \mu\text{g ml}^{-1}$ anti-CD3 (145.2C11) antibody and washed with PBS before plating cells. Counts from B or T cells alone were $<5\%$ that of co-stimulated cells. Proliferation was measured by [^3H]thymidine incorporation ($0.5 \mu\text{Ci}$ per well) during the last 16 h of a 64-h culture (Canberra-Packard Matrix 9600 Direct beta counter). All assays were performed in triplicate with less than 20% variance between wells. Results are representative of three (a, b) or two (c) independent experiments.

ity, in terms of tempo, size and quality of the response. This makes CD19 a potential target for therapy in antibody-dependent autoimmune diseases.

In conclusion, although expressed much earlier in B-cell development, the role of CD19 becomes evident when B cells respond to, and are selected by, antigen. The B-1 cell deficiency in CD19-deficient mice may thus reflect inefficient selection of these cells by internal antigens. In contrast, conventional B cells may not require antigen for their maintenance. □

27. Torres, R. & Kühn, R. *The Cologne Guide to Gene Targeting* (Garland, New York, in the press).
28. Wells, S. M., Kantor, A. B. & Stall, A. M. *J. Immunol.* **153**, 5503–5515 (1994).
29. Roes, J. & Rajewsky, K. *J. exp. Med.* **177**, 45–55 (1993).

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1. Carter, R. H. & Fearon, D. T. *Science* **256**, 105–107 (1992).
2. Hardy, R. R. & Hayakawa, K. *Adv. Immunol.* **55**, 297–339 (1994).
3. Fearon, D. T. *Curr. Opin. Immunol.* **5**, 341–348 (1993).
4. Tedder, T. F., Zhou, L.-J. & Engel, P. *Immun. Today* **15**, 437–442 (1994).
5. Mercolino, T. J., Arnold, L. W., Hawkins, L. A. & Haughton, G. J. *exp. Med.* **168**, 687–698 (1988).
6. Bradbury, L. E., Kansas, G. S., Levy, S., Evans, R. L. & Tedder, T. F. *J. Immunol.* **149**, 2841–2850 (1992).
7. Matsumoto, A. K. *et al. J. exp. Med.* **173**, 55–64 (1991).
8. Carter, R. H., Spycher, M. O., Ng, Y. C., Hoffman, R. & Fearon, D. T. *J. Immunol.* **141**, 457–463 (1988).
9. Tsokos, G. C., Lambris, J. D., Finkelman, F. D., Anastassiou, E. D. & June, C. H. *J. Immunol.* **144**, 1640–1645 (1990).
10. Förster, I. & Rajewsky, K. *Eur. J. Immunol.* **17**, 521–528 (1987).
11. McHeyzer-Williams, M. G., McLean, M. J., Lalor, P. A. & Nossal, G. J. *J. exp. Med.* **178**, 295–307 (1993).
12. Reth, M., Hammerling, G. J. & Rajewsky, K. *Eur. J. Immunol.* **8**, 393–400 (1978).
13. Cumano, A. & Rajewsky, K. *EMBO J.* **2**, 2459–2468 (1986).
14. Jacob, J., Kelsoe, G., Rajewsky, K. & Weiss, U. *Nature* **354**, 389–392 (1991).
15. Liu, Y. & Janeway, C. Jr. *Int. Immunol.* **3**, 323–332 (1991).
16. Heyman, B. in *New Aspects of Complement Structure and Function* (ed. Erdei, A.) 59–72 (Landes Co., London, 1994).
17. Hebel, T., Ahearn, J. M. & Fearon, D. T. *Science* **254**, 102–105 (1991).
18. Heyman, B., Wiersma, E. J. & Kinoshita, T. *J. exp. Med.* **172**, 665–668 (1990).
19. Thyphronitis, G. *et al. J. Immunol.* **147**, 224–230 (1991).
20. Carter, R. H. & Fearon, D. T. *J. Immunol.* **143**, 1755–1760 (1989).
21. Bohnsack, J. F. & Cooper, N. R. *J. Immunol.* **141**, 2569–2576 (1988).
22. Melchers, F., Erdei, A., Schulz, T. & Dierich, M. P. *Nature* **317**, 264–267 (1985).
23. van Noesel, C. J. M., Lankester, A. C. & van Lier, R. A. W. *Immun. Today* **14**, 8–11 (1993).
24. Zhou, L. J., Ord, D. C., Omori, S. A. & Tedder, T. F. *Immunogenetics* **35**, 102–111 (1992).
25. Jung, S., Rajewsky, K. & Radbruch, A. *Science* **259**, 984–987 (1993).
26. Gu, H., Marth, J. D., Orban, P. C., Mossman, H. & Rajewsky, K. *Science* **265**, 103–106 (1994).

Allelic exclusion of Ly49-family genes encoding class I MHC-specific receptors on NK cells

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An important feature of natural killer (NK) cell activity is the lysis of cells that have extinguished expression of some or all class I major histocompatibility (MHC) molecules^{1–7}. Accordingly, the Ly49A NK-cell antigen receptor has been shown to deliver an inhibitory signal to NK cells on encounter with D^d or D^k class I MHC on target cells⁴. Ly49A belongs to a family of eight or more highly related, tightly linked genes^{2,8–10}. Expression of Ly49A and Ly49C, another member of the Ly49 family with distinct MHC

specificity, define subpopulations of NK cells that are only partly overlapping¹⁰⁻¹². The mechanisms regulating the expression of Ly49 family members are unknown. We show here that the Ly49A and Ly49C NK-cell receptors are each subject to allelic exclusion. Because Ly49 genes are not thought to undergo DNA rearrangement^{13,14}, allelic exclusion of Ly49 genes could involve a mechanism distinct from that used by B and T lymphocytes^{15,16} and is likely to play an important role in the genesis of a putative NK-cell repertoire specific for class I molecules.

The Ly49A NK-cell receptor is expressed by 15–20% of C57BL/6 (B6) NK cells, based on analysis with the two monoclonal antibodies A1 (ref. 17) and JR9-318 (ref. 18; Fig. 1). Both monoclonal antibodies recognize the Ly49A protein in B6 mice, as indicated by preclearing immunoprecipitation experiments¹⁸, and by staining of completely overlapping NK-cell subsets (Fig. 1). In the BALB/c strain, JR9-318 stains 4–10% of NK cells, whereas A1 does not stain any cells (Fig. 1).

To evaluate the allele specificity of these monoclonal antibodies, complementary DNAs corresponding to putative *Ly49A* alleles were cloned from B6 and BALB/c interleukin-2 cultured

adherent lymphokine activated killer (A-LAK) cell RNA by reverse transcription and polymerase chain reaction (RT-PCR) using primers specific for *Ly49A*. The B6 and BALB/c *Ly49A* cDNA sequences were 99% identical (Fig. 2 legend). The BALB/c sequence contained a diagnostic *StuI* site, absent in the B6 sequence, which was confirmed with three non-overlapping *Ly49A*-specific PCR primer sets. These data indicate that the sequence containing *StuI* was the only *Ly49A*-like sequence expressed by the BALB/c strain (Fig. 2a and results not shown). Finally, COS cells transiently transfected with the B6 *Ly49A* cDNA stained with both the JR9-318 and A1 monoclonal antibodies, whereas COS cells transfected with the BALB/c cDNA stained only with JR9-318 (results not shown). These results strongly suggest that the two sequences represent alleles of *Ly49A*, and that A1 reacts specifically with the B6 allele-product, whereas JR9-318 reacts with both the B6 and BALB/c allele-products.

To assess whether both *Ly49A* alleles are expressed in individual NK cells from *Ly49A* heterozygous mice, freshly isolated NK cells from (BALB/c × B6)_{F1} (CB6F₁) mice were stained with A1 and JR9-318. Approximately half of the cells that stained with JR9-318 did not stain with the A1 antibody, suggesting that these cells express the BALB/c but not the B6 *Ly49A* allele (Fig. 1). The JR9-318⁺A1⁺ cells do express the B6 allele, but the staining data do not address whether they also express the BALB/c allele. These observations were confirmed in A-LAK cells derived from CB6F₁ (Fig. 1) and several other *F1* mice (not shown). *Ly49A*-specific PCR was then used to determine the origin of the *Ly49A* transcripts in sorted populations of JR9-318⁺A1⁺ and JR9-318⁺A1[−] CB6F₁ A-LAK cells. Whereas *Ly49A* transcripts detected in the former population were derived only from the B6 allele, transcripts in the latter population were derived only from the BALB/c allele of *Ly49A* (Fig. 2a). These data indicate that the two *Ly49A* alleles are expressed predominantly or exclusively in different cell populations and that the differential expression of alleles is manifested at the level of messenger RNA.

We subsequently assessed whether expression of *Ly49C*, detected in B6 mice with the monoclonal antibody SW5E6 (refs 11, 12, 19), is also subject to allelic exclusion. SW5E6 fails to stain A-LAK cells derived from 129/Sv mice (Fig. 3) and may therefore not recognize the 129/Sv *Ly49C* allele-product¹¹. The B6 and 129/Sv *Ly49C* cDNA sequences, isolated by RT-PCR, were 98% identical (Fig. 2 legend). As expected, SW5E6 reacted only with COS cells transiently transfected with the B6-derived *Ly49C* cDNA clone and not with those transfected with the 129/Sv-derived clone (results not shown). Sorted SW5E6⁺ A-LAK cells from (129/Sv × B6)_{F1} mice expressed predominantly the B6 *Ly49C* sequence, whereas the SW5E6[−] population expressed predominantly the 129/Sv sequence (Fig. 2b). Allelic exclusion may therefore be a feature shared by the entire Ly49 multigene family.

Although Ly49A and Ly49C define largely non-overlapping populations, the two receptors are co-expressed by a minor subset of A-LAK cells (3.0% of total in B6 mice) (Fig. 3) and fresh NK cells¹⁰. Allelic exclusion in NK cells co-expressing Ly49A and Ly49C could therefore be coordinate or independent. Thus *Ly49C* mRNA expression in *Ly49A*[−] cells was analysed using the sorted *Ly49A*^{B6} and *Ly49A*^{BALB/c} populations from the CB6F₁ mice described in Fig. 1. Although the JR9-318⁺A1[−] population expresses solely the *Ly49A*^{B6} allele (Fig. 2a), approximately equal amounts of *Ly49C*^{B6} and *Ly49C*^{BALB/c} transcripts were detected in this cell population (Fig. 4). Similarly, although the JR9-318⁺A1⁺ population expressed only the *Ly49A*^{BALB/c} allele (Fig. 2a), an equivalent expression of both *Ly49C* alleles was also detected in this population (Fig. 4). Because *Ly49C* is allelically excluded (Fig. 2b), these results suggest that allelic exclusion of *Ly49A* and *Ly49C* occurs independently, rather than involving the activation (or inactivation) of the entire array of Ly49 genes on a given chromosome.

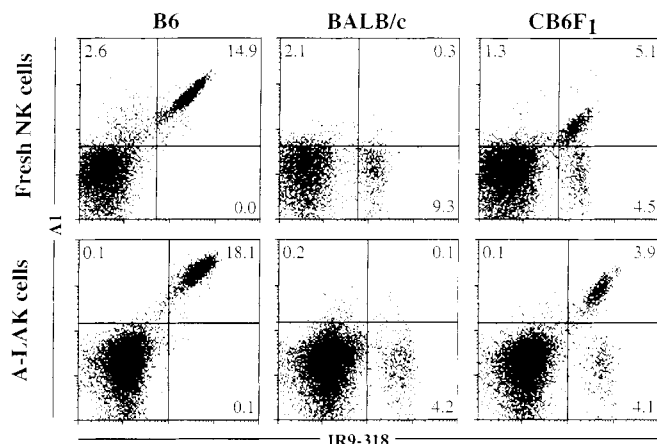
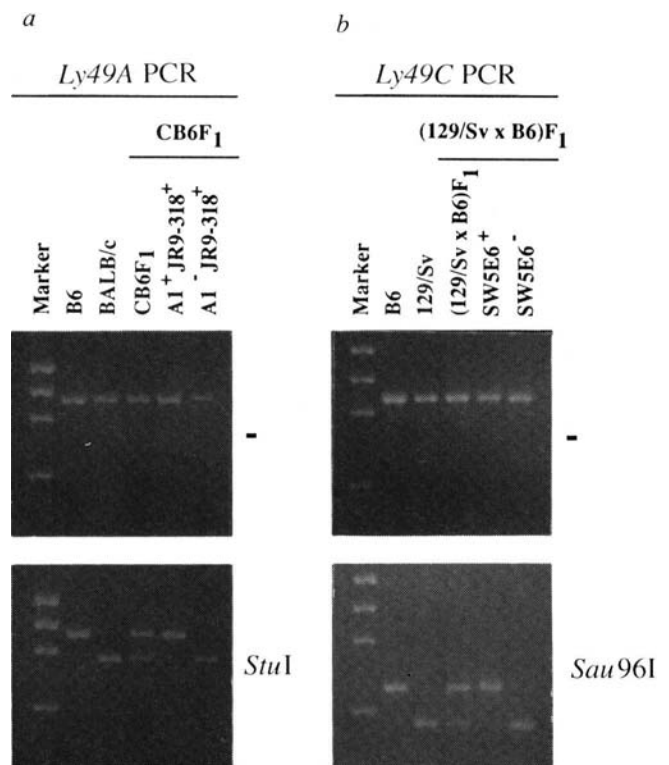


FIG. 1 Expression of Ly49A alleles in NK cells. Freshly isolated splenic NK cells (upper panel) or A-LAK cells (lower panel) from C57BL/6 (B6), BALB/c and (C57BL/6 × BALB/c)_{F1} (CB6F₁) mice were analysed for expression of *Ly49A* alleles. Monoclonal antibody (mAb) A1 is specific for the product of the B6 *Ly49A* allele, whereas mAb JR9-318 recognizes both the B6 and BALB/c allele products. Somewhat lower *Ly49A* expression levels are observed in H-2^d mice²¹. Freshly isolated NK cells or A-LAK cells from CB6F₁ mice show a staining pattern that is consistent with mutually exclusive expression of the two *Ly49A* alleles. These observations were confirmed in several other *F1* mice, such as (B6 × BALB.B)_{F1}, (DBA/2 × B6)_{F1} and (CBA/J × B6)_{F1} mice (not shown). Identical staining results were also obtained with the *Ly49A*-specific monoclonal antibodies YE1-32 and YE1-48 (refs 10, 22) instead of JR9-318.

METHODS. Splenic NK cells were enriched by passage through nylon wool followed by complement-mediated depletion of CD4⁺ (using mAb RL172 (ref. 23)) and CD8⁺ (mAb AD4[15] (ref. 24)) T cells. A-LAK cells were prepared as previously described²⁵ and harvested at day 10. 5×10^5 to 10^6 cells were stained with anti-mouse IgG labelled with phycoerythrin (PE) (Southern Biotechnology, Birmingham, Alabama) followed by 2.4G2 (rat anti-FcR)²⁶ hybridoma supernatant plus mouse IgG to reduce non-specific staining. After washing, the cells were incubated with a mixture of fluorescein isothiocyanate (FITC)-labelled JR9-318 (mouse anti-*Ly49A*)¹⁸ and biotinylated A1 (mouse anti-*Ly49A*^{B6})¹⁷ followed by Streptavidin Tricolor (Caltag, San Francisco) and PE-labelled anti-CD3 (Pharmingen, San Diego). 2×10^4 to 10^5 events were analysed on an XL flow cytometer (Coulter, Hialeah, Florida), using forward and side scatter to exclude non-viable cells. Analysis was performed with the WinMDI software (J. Trotter, Salk Institute) excluding residual CD3⁺ and Ig⁺ cells from the data analysis. With this method, the fresh CD3⁺ Ig⁺ cells from B6 spleens were >90% NK1.1⁺, as detected with the B6 allele-specific mAb PK136 (ref. 27). Total B6-derived A-LAK cells were usually >95% NK1.1⁺.

FIG. 2 Expression of *Ly49A* and *Ly49C* (5E6) mRNAs in sorted A-LAK cell populations. **a**, Total A-LAK cells were obtained from B6, BALB/c and CB6F₁ mice. The A1⁺JR9-318⁺ and A1⁻JR9-318⁻ sub-populations were sorted from CB6F₁-derived A-LAK cells. RT-PCR with *Ly49A*-specific primers was used to amplify *Ly49A* cDNA. The two *Ly49A* cDNAs were discriminated by digestion with *Stu*I, which cleaves the *Ly49A* PCR product derived from BALB/c but not that derived from B6. **b**, Total A-LAK cells were obtained from B6, 129/Sv and (129/Sv × B6)_F₁ mice. SW5E6⁺ and SW5E6⁻ sub-populations were sorted from (129/Sv × B6)_F₁-derived A-LAK cells and RT-PCR with *Ly49C* (5E6)-specific primers was used to amplify *Ly49C* cDNA from these subsets. The two *Ly49C* PCR products were discriminated with *Sau*96I, which cleaves the *Ly49C* PCR product derived from 129/Sv but not that derived from B6.

METHODS. cDNAs corresponding to *Ly49A* alleles from the B6 and BALB/c strains were obtained by RT-PCR from RNA isolated from d10 A-LAK cells using conditions described before²⁸ with primers containing an *Eco*RI site¹³: 5' primer (sense) CATCGAATTCAACCAGAAAAAGCCAACTT; 3' primer (antisense) CATCGAATTCAATGAGGAATTTATCCAG. The PCR product was cloned into the *Eco*RI site of the Bluescript SK⁺ polylinker. Sequence analysis of several *Ly49A* clones indicated that there is one nucleotide difference between the published *Ly49A* sequence¹³ and our B6 *Ly49A* sequence, which replaces the methionine at position 106 with a isoleucine. There are 7 nucleotide substitutions between our *Ly49A* B6 and BALB/c sequences (99% identity). This identity score exceeds homologies with any other *Ly49* family member (<81%)^{9,10}. The nucleotide substitutions introduce 5 amino acid changes (98% identity), 4 of which are located in the extracellular portion of the molecule. Again, the amino acid identity score exceeds any other identity with *Ly49* family members (<78%)^{9,10}. This was determined by sequence alignments by MacVector using the DNA identity matrix. The *Ly49A* BALB/c sequence will be submitted to GenBank. CB6F₁-derived A-LAK cells were harvested at day 10. After staining as described in the legend to Fig. 1, A1⁺JR9-318⁺ (4.0% of total) and A1⁻JR9-318⁻ (4.3%) A-LAK cell populations were sorted to a purity of >97% using an EPICS cell sorter (Coulter). Total cellular RNA prepared from these cells was subjected to RT-PCR using primers specific for both *Ly49A* alleles: 5' primer (sense) ACCAGAACCACTTCTTG(C,A)TAGC; 3' primer (antisense) CATCGAATTCAAAACACTACTTGTTCGCAAGG, containing an *Eco*RI site. 5 µl of the PCR reaction mixture was then digested with various restriction enzymes, which homogeneously cut all PCR products (for example, *Xho*I) (not shown) or differentially cut the B6 and BALB/c *Ly49A*-derived PCR products (for example, *A*/wI (not shown) and *Stu*I). The fragments were separated on a 3% NuSieve (FMC BioProducts, Rockland, Maine) agarose gel and revealed under ultraviolet after staining with ethidium bromide. The marker fragment sizes are 1.4, 1.1, 0.9 and 0.6 kb. The cDNAs corresponding to the putative *Ly49C* (previously termed 5E6) alleles from the 129/Sv and B6 strains of mice were amplified by RT-PCR as described above, using primers containing an *Eco*RI site^{12,19}. 5' primer (sense) CATCGAATTCGAGGAGGGGCA-GAAATCA; 3' primer (antisense) CATCGAATTCATGTTCTTTAACTCTGG. The PCR product was cloned into the *Eco*RI site of the Bluescript SK⁺ polylinker. Sequence analysis of *Ly49C* clones and direct sequencing of the PCR product²⁹ indicated that there are 17 nucleotide substitutions (98% identity) between our *Ly49C* of B6 and 129/Sv sequences. There are 32 and 33 differences (96% identity) between our two *Ly49C* sequences compared with the published *Ly49C* sequence¹⁰. Because



the latter was isolated from a (B6 × CBA/J)_F₁ cDNA library we inferred that it corresponds to the CBA/J *Ly49C* sequence. This was confirmed by amplifying and sequencing *Ly49C* from CBA/J A-LAK cDNA (results not shown). At the amino acid level the B6 *Ly49C* allele is 92% and the 129/Sv *Ly49C* allele is 93% identical with the published, that is, the CBA/J-derived, *Ly49C* sequence. The B6 and 129/Sv *Ly49C* alleles are 96% identical. These percentages exceed identities with any other *Ly49* family member at the nucleotide (92%) or amino acid level (86%)^{9,10}. Alignments were done as described above. The *Ly49C* B6 and 129/Sv sequences will be submitted to GenBank. A-LAK cells derived from (129/Sv × B6)_F₁ mice were harvested at day 10. The cells were first incubated with 2.4G2 hybridoma supernatant to reduce non-specific staining, followed by incubation with FITC-labelled SW5E6 (mouse anti-*Ly49C*)¹¹. *Ly49C*⁺ (16.2% of total) and *Ly49C*⁻ (83.8%) populations were sorted to a purity of >97%. Total cellular RNA prepared from these cells was then subjected to RT-PCR with the 5' primer described above and a 3' primer specific for *Ly49C*: 3' primer (antisense) GTAGTAGGGAATATTACAGTC. 5 µl of the PCR reaction mixture was digested with various restriction enzymes, which homogeneously cut all PCR products (for example, *Xba*I) (not shown) or differentially cut the B6 and 129/Sv *Ly49C*-derived PCR products (for example, *M*scl (not shown) and *Sau*96I). The fragments were analysed as described above.

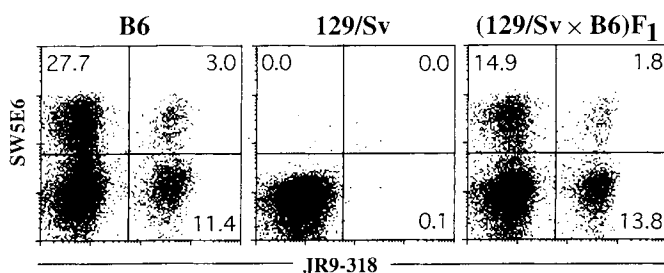


FIG. 3 Co-expression of *Ly49A* and *Ly49C* (5E6). A subset of B6-derived A-LAK cells stained with both the JR9-318 and SW5E6 mAbs, whereas 129/Sv derived A-LAK cells failed to stain with either mAb. Cells that co-stained with the two mAbs were readily detected among _F₁ A-LAK cells, demonstrating that in individual cells expression of *Ly49A* and *Ly49C* can occur from the same (B6) chromosome. Although the formal mapping of the *Ly49C* to chromosome 6 has not been reported, the high degree of nucleotide sequence identity (81%) with *Ly49A* (ref. 10) and the fact that the multiple bands in low-stringency Southern blots with an *Ly49A* probe co-segregate³⁰ strongly suggest that *Ly49C* is part of the tightly linked *Ly49* family encoded on chromosome 6.

METHODS. Day 7 A-LAK cells were incubated with 2.4G2 hybridoma supernatant, followed by a mixture of FITC-labelled JR9-318 and biotinylated SW5E6 followed by Streptavidin-Tricolor (Caltag) plus PE-labelled anti-CD3 (Pharmingen). 2 × 10⁴ cells were analysed as described above, excluding residual CD3⁺ cells from the data analysis.

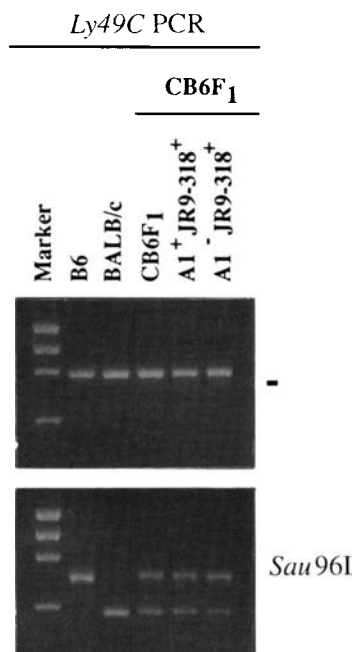


FIG. 4 NK cells that express only one *Ly49A* allele can express *Ly49C* encoded by the same or opposite chromosome. RT-PCR and restriction digests were used to assess expression of *Ly49C* transcripts among sorted *Ly49A^{B6}* and *Ly49A^{BALB/c}* A-LAK cell populations derived from, CB6F₁ mice. Restriction enzyme digestion with *Sau96I* was used to discriminate between the BALB/c and B6 *Ly49C*-derived PCR products. FMF analysis revealed that the *Ly49A^{BALB/c}Ly49C^{B6}* population exhibited allelic exclusion of *Ly49A*, as it was composed of equal numbers of JR9-318⁺A1⁺ and JR9-318⁺A1⁻ cells (not shown). METHODS. cDNA from sorted A1⁺JR9-318⁺ (*Ly49A^{B6}*) and A1⁻JR9-318⁻ (*Ly49A^{BALB/c}*) populations described in Figs 1 and 2 was subjected to PCR specific for *Ly49C* as described in the legend to Fig. 2, using primers specific for *Ly49C*: 5' primer (sense) CAGACTTCTGTACTCC-CACG; 3' primer (antisense) AACCTCAGGTGGAAAGTTAATCAGGG. 5 µl of the PCR reaction mixture was digested as described in the legend to Fig. 2 for the *Ly49C* PCR. The fragments were analysed as described above.

Allelic exclusion of *Ly49* genes presumably reflects a process that limits the number of different specific receptors expressed by individual NK cells, as is presumed for allelic exclusion of T and B cell receptors^{15,16} as well as of odorant receptors²⁰. The expression of one or a small number of MHC-specific receptors by individual NK cells would allow the lysis of cells that extinguish some but not all class I molecules, consistent with the presumed function of NK cells in the lysis of autologous cells that extinguish class I gene expression owing to mutation, transformation or infection. Allelic exclusion might contribute to limiting the number of MHC-specific receptors if alleles of individual *Ly49* genes sometimes encode distinct class I specificities. A difficulty with this model is that coexpression of NK receptors with different specificities is clearly tolerated, as shown by the presence of *Ly49A⁺C⁺* cells. An alternative model is that a *cis*-acting, stochastic mechanism imparts a specific probability of stable activation to each *Ly49* gene/allele in individual NK clones. Whereas at a given locus most cells would express only one allele, a small fraction of cells (below detection in the present experiments) could express both. Co-expression of several different family members would occur on some cells, but many other cells would express only one or a few receptors. The expression of multiple inhibitory receptors by NK cells may represent less of a threat to the animal than would the expression of multiple activating receptors, as in B and T cells. Experiments are in progress to determine whether

the mechanism that imposes allelic exclusion of *Ly49* genes is stochastic or regulated. □

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- Karre, K. *Semin. Immun.* **5**, 127–145 (1993).
- Yokoyama, W. M. & Seaman, W. E. *A. Rev. Immun.* **11**, 613–635 (1993).
- Raulet, D., Correa, I., Corral, L., Dorfman, J. & Wu, M.-F. *Semin. Immun.* **7** (in the press).
- Karlhofer, F. M., Ribaud, R. K. & Yokoyama, W. M. *Nature* **358**, 66–70 (1992).
- Moretta, A. *et al. J. exp. Med.* **178**, 597–604 (1993).
- Moretta, A. *et al. J. exp. Med.* **180**, 545–555 (1994).
- Litwin, V., Gumperz, J., Parham, P., Phillips, J. & Lanier, L. L. *J. exp. Med.* **180**, 537–543 (1994).
- Yokoyama, W. M. *et al. J. Immun.* **147**, 3229–3236 (1991).
- Smith, H. R. C., Karlhofer, F. M. & Yokoyama, W. M. *J. Immun.* **153**, 1068–1079 (1994).
- Brennan, J., Mager, D., Jefferies, W. & Takei, F. *J. exp. Med.* **180**, 2287–2295 (1994).
- Sentman, C. L., Hackett, J. Jr, Kumar, V. & Bennett, M. J. *J. exp. Med.* **170**, 191–202 (1989).
- Stoneman, E., Kumar, V., Bennett, M., Matthew, P. A. (Abstr.) *J. Immun.* **150**, 1465a (1993).
- Yokoyama, W. M., Jacobs, L. B., Kanagawa, O., Shevach, E. M. & Cohen, D. I. *J. Immun.* **143**, 1379–1386 (1989).
- Chan, P. Y. & Takei, F. *J. Immun.* **142**, 1727–1736 (1989).
- Malissen, M. *et al. Immun. Today* **13**, 315–322 (1992).
- Storb, U. A. *Rev. Immun.* **5**, 151–174 (1987).
- Nagasawa, R. *et al. J. Immun.* **138**, 815–824 (1987).
- Roland, J. & Cazenave, P. A. *Int. Immun.* **4**, 699–706 (1992).
- Wong, S., Freeman, J. D., Kelleher, C., Mager, D. & Takei, F. *J. Immun.* **147**, 1417–1423 (1991).
- Chess, A., Simon, I., Cedar, H. & Axel, R. *Cell* **78**, 823–834 (1994).
- Karlhofer, F. M., Hunziker, R., Reichlin, A., Margulies, D. H. & Yokoyama, W. M. *J. Immun.* **153**, 2407–2416 (1994).
- Takei, F. *J. Immun.* **130**, 2794–2799 (1983).
- Ceredig, R., Lowenthal, J., Nabholz, M. & MacDonald, H. R. *Nature* **314**, 98–100 (1985).
- Raulet, D. H., Gottlieb, P. & Bevan, M. J. *J. Immun.* **125**, 1136–1143 (1980).
- Correa, I., Corral, L. & Raulet, D. H. *Eur. J. Immun.* **24**, 1323–1331 (1994).
- Unkeless, J. C. *J. exp. Med.* **145**, 931–945 (1977).
- Koo, G. C. & Peppard, J. R. *Hybridoma* **3**, 301–303 (1984).
- Held, W. *et al. J. exp. Med.* **177**, 359–366 (1993).
- Casanova, J. L. *Meth. molec. Biol.* **23**, 191–197 (1993).
- Yokoyama, W. M., Kehn, P. J., Cohen, D. I. & Shevach, E. M. *J. Immun.* **145**, 2353–2358 (1990).

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Requirement for tyrosine phosphorylation of Cdk4 in G1 arrest induced by ultraviolet irradiation

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EXPOSURE to ultraviolet light arrests the function of mammalian fibroblasts in the G1 phase of the cell cycle, as well as the S and G2 phases. Although p21, an inhibitor of cyclin-dependent kinase (Cdk) that is induced by DNA damage may partly account for the arrest in G1 (ref. 1), the mechanism is little understood. Here we show that tyrosine phosphorylation of Cdk4 is required for this arrest. In rat fibroblast, Cdk4 is tyrosine-phosphorylated during G1 progression, and its dephosphorylation is required for S phase. When cells are ultraviolet-irradiated, their arrest in G1 is accompanied by an increase in phosphorylation level. Conversely, cells expressing unphosphorylatable Cdk4^{F17} fail to arrest in G1, and suffer significantly elevated chromosomal aberrations and cell death.

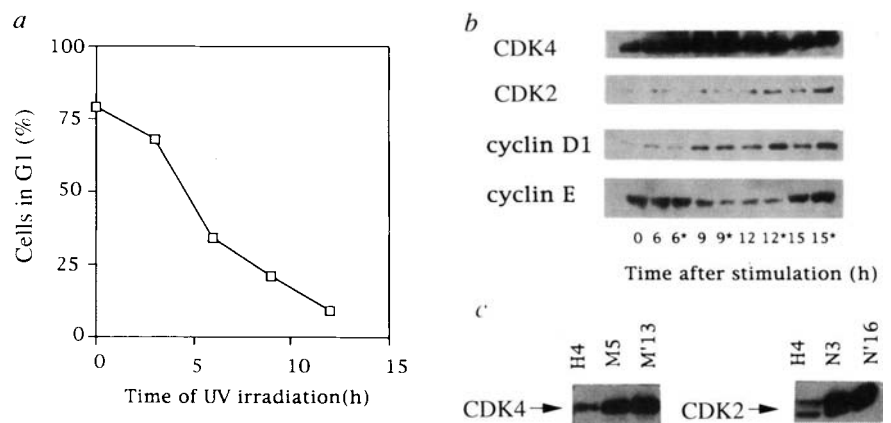
TABLE 1 Chromosomal aberrations induced by UV irradiation

Cell line	UV dose (J m ⁻²)	Cells analysed	Chromosomal aberrations	Chromosomal aberrations per cell	Breaks	Exchanges
Control cells						
NRK	0	196	4	0.020	3	1
	7.5	205	26	0.127	18	8
H4	0	185	2	0.011	2	0
	7.5	200	21	0.105	15	6
Wild-type Cdk2-expressing cells						
N3	0	230	6	0.026	6	0
	7.5	201	21	0.104	19	2
N4	0	185	1	0.005	0	1
	7.5	196	8	0.041	5	3
Mutated type Cdk2-expressing cells						
N'16	0	216	1	0.005	1	0
	7.5	203	12	0.059	8	4
N'19	0	173	3	0.017	1	2
	7.5	196	8	0.041	7	1
Wild-type Cdk4-expressing cells						
M2	0	206	3	0.015	3	0
	7.5	206	21	0.102	15	6
M5	0	200	7	0.035	6	1
	7.5	200	33	0.165	26	7
M5*	0	210	5	0.024	4	1
	7.5	203	152	0.749	91	61
Mutated type Cdk4-expressing cells						
M'8	0	208	1	0.005	1	0
	7.5	204	151	0.740	95	56
M'13	0	200	5	0.025	3	2
	7.5	200	188	0.940	124	64
M'13*	0	215	6	0.028	4	2
	7.5	208	180	0.865	109	71

Cells were synchronized, irradiated with UV (7.5 J m⁻²) or mock-irradiated at 2 h or 12 h (marked by asterisk) poststimulation and cultured for 24 h. Colcemid (25 ng M⁻¹) was added to the culture, which was further incubated for 4 h. Chromosomal spread samples were prepared by the standard method and microscopically examined.

FIG. 1 a, Time dependence of UV-induced G1 arrest. NRK cells were synchronized in G1 by arrest and stimulation, irradiated with UV at 0, 3, 6, 9 or 12 h poststimulation or mock-irradiated, collected at 22 h and analysed by flow cytometry. Each point represents percentage net G1-arrested population (percentage G1 population of irradiated cells minus percentage G1 population (5%) of mock-irradiated cells). b, UV-irradiation does not affect the levels of cyclins and Cdk's. Cells were synchronized UV-irradiated or mock-irradiated at 2 h poststimulation and incubated for the indicated time (0, 6, 9, 12, 15 h). Extracts from unirradiated and UV-irradiated (indicated by asterisks) cells were electrophoresed on 12% SDS-polyacrylamide gels and immunoblotted. c, Levels of Cdk4 and Cdk2 proteins in cell clones expressing an empty vector (H4), Cdk4 (M5), Cdk4^{F17} (M'13), Cdk2 (N3) or Cdk2^{F15} (N'16). Western blots were performed for extracts prepared from asynchronous cultures of these cells.

METHODS. a, The normal rat kidney cell line NRK-49F (American Type Culture Collection) was maintained as described⁴. Cells grown to confluence were incubated in DMEM containing 0.05% FCS for 48 h and then released from arrest by replating at a density of 2×10^5 cells per 10-cm dish in DMEM containing 5% FCS. At 0, 3, 6, 9 or 12 h poststimulation, cells were irradiated with 7.5 J m⁻² of UV under a low-pressure mercury lamp and incubated for 22 h. Nuclei were then prepared from the cells, stained with propidium iodide using the Cycle Test (Becton-Dickinson, CA) and analysed with a fluorescence-activated cell analyser as described previously²³. b, Cell cycling was initiated by replating G1-arrested cells under the same conditions as above. Cells were UV(7.5 J m⁻²)-irradiated or mock-irradiated at 2 h poststimulation and incubated for the indicated time. Cell extracts were then prepared by lysing with ice-cold modified RIPA buffer (10 mM Tris-HCl, pH 7.5), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1 mM



PMSF, 4 mg ml⁻¹ leupeptin, pepstatin and aprotinin, 1 mM sodium pyrophosphate, 10 mM sodium vanadate and 25 mM sodium fluoride). For western blots, extracts were electrophoresed on 12% SDS-polyacrylamide gels, transferred to Immobilon membranes (Millipore), probed with antibodies to Cdk4 (C-22), Cdk2 (M2), cyclin D1 (PRAD1) or cyclin E (C-19) (Santa Cruz Biotechnology), and detected for signals with the ECL system (Amersham). c, Oligonucleotide-mediated mutagenesis of the murine Cdk4 (ref. 5) and human Cdk2 (ref. 15) complementary DNA was performed using the Altered Sites Mutagenesis system (Promega). Wild-type and mutant Cdk4 and Cdk2, subcloned into PEF, hygromycin containing the EF1 α promoter²⁴ and the hygromycin selectable marker gene, were transfected into NRK as described²⁵. Drug-resistant colonies were selected, and those expressing the transfected genes were determined by western blot with appropriate antibodies, as described above.

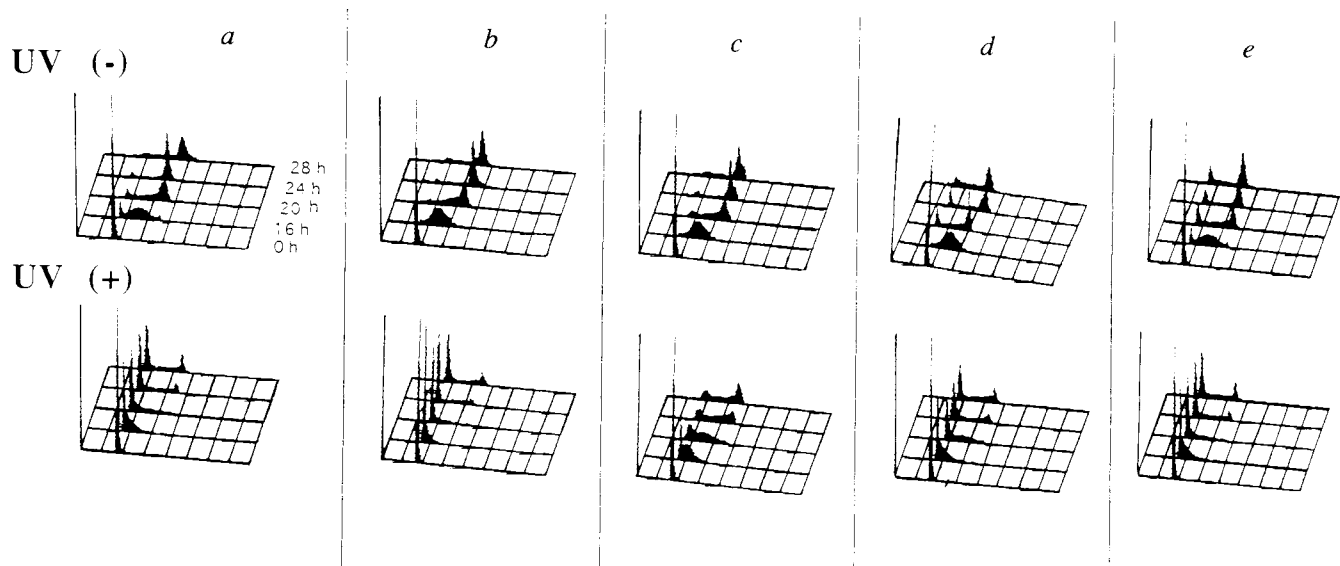


FIG. 2 Cells expressing Cdk4^{F17} are unable to arrest in G1 after UV irradiation. H4 (a), M5 (b), M'13 (c), N3 (d) and N'16 (e) were synchronized, UV-irradiated or mock-irradiated at 2 h poststimulation, and incubated further in the presence of colcemid (25 ng ml⁻¹). Cells were

collected at 0, 16, 20, 24 and 28 h, and analysed for their cell cycle progression by flow cytometry.

METHODS. Cell cycling, irradiation and FACS analysis were as described in Fig. 1a.

The normal rat kidney fibroblast line NRK-49F responds to ultraviolet irradiation by an arrest in cell cycling (Fig. 1a). These cells have an active p53, as indicated by gel shift assay using the whole extracts of cells treated with or without methylmethane sulphonate and the p53-responsive element of the human *GADD45* gene as a probe² (data not shown). When arrested in G1 by serum depletion and induced to resume cell cycling, NRK cells began to enter the S phase at 12 h, and most were in mid-S phase at 15–16 h (Fig. 2), before returning to G1 at 24 h. Ultraviolet irradiation arrested virtually all of the cells in G1, S or G2 until 28 h, but exposure in early G1 most effectively induced arrests in G1. When irradiated within 3 h, >70% of the cells arrested in G1, whereas <20% arrested when irradiated after 9 h. Under these conditions, 95% of mock-irradiated cells entered the S phase and continued cell cycling.

Cdk4, Cdk2, cyclin D1 and cyclin E are important for the start of cell cycle³. In NRK, Cdk4 was constitutively expressed whereas cyclin D1, Cdk2 and cyclin E induced at mid-G1, at the G1/S boundary, and at early G1 and S, respectively. Ultraviolet irradiation did not affect their expression (Fig. 1b).

In this cell line, Cdc25A is induced at the same time as cyclin D1 (refs 4, 5). Cdc25A is one of the three Cdc25 phosphatase homologues identified in mammals, and is required for the cell cycle 'start'^{4,6}. *In vitro* and in yeast it can activate Cdc2 and related kinases by dephosphorylating at Y15 or a corresponding site^{4,6}. In G2, Cdc2 is mainly regulated by this tyrosine phosphorylation and dephosphorylation which are catalysed by Wee1 kinase and Cdc25 phosphatase(s)^{7,8}; this regulation constitutes a key mechanism for DNA damage-responsive, G2 checkpoint arrest^{9–12}. These facts raise an interesting possibility that a Cdc2-related cell-cycle 'start' kinase(s), such as Cdk4 or Cdk2, might similarly be regulated, and that such regulation might similarly be involved in damage-responsive checkpoint arrest. Cdk2 is known to be phosphorylated at Y15 during cell cycling^{13,14}.

To investigate this possibility, unphosphorylatable mutant Cdk4 and Cdk2 were constructed by replacing their Y17 and Y15, which corresponded to the regulatory Y15 of Cdc2 (refs 5, 15), with phenylalanine. NRK was then transfected with Cdk4, Cdk4^{F17}, Cdk2, Cdk2^{F15} (or none of these) inserted in an expression vector, and colonies constitutively expressing these transfected genes were selected. Several expressors were isolated, and those named M5, M'13, N3 and N'16, together with H4, an empty vector expressor, were extensively characterized as repre-

sentatives. These clones expressed three- to fivefold higher levels of transfected Cdk4 or Cdk2 than endogenous proteins (Fig. 1c). Their growth rate and cell morphology were, however, similar to those of parent NRK cells.

To determine a possible defect in DNA damage-responsive G1-arrest ability, they were synchronized as above, irradiated with ultraviolet at 2 h poststimulation, and monitored for their cell cycling in the presence of colcemid which blocked G1 re-entry (Fig. 2). When irradiated, all arrested in G1 to the same extent except M'13, the Cdk4^{F17} expressor, which failed to arrest and entered the S phase as normal. Nevertheless, M'13 retained the active p53 protein, as confirmed by the same gel shift assay, and a full ability to arrest in S and G2 (Fig. 2 and data not shown). When exponentially growing NRK was similarly irradiated, a much smaller population underwent G1-arrest. Again, M'13 failed to arrest (data not shown). These results were confirmed with 5–6 other independent clones expressing mutant or wild-type Cdk4 (data not shown).

Because Cdc25A is required for the cell cycle 'start', microinjection of a specific anti-Cdc25A antibody into NRK cells blocks their entry into S (ref. 4). Immunodepletion of Cdc25A indeed blocked M5, but not M'13 (Fig. 3a). Identical results were obtained with other Cdk4 or Cdk4^{F17} expressors, respectively. We concluded that the cells expressing unphosphorylatable Cdk4^{F17} no longer required Cdc25A, indicating that a critical target for Cdc25A was Cdk4, and that Cdk4 might be regulated by Y17 phosphorylation and dephosphorylation during G1.

To confirm phosphorylation and its regulation, G1-arrested M5 and M'13 cells were induced for cell cycling with or without subsequent ultraviolet irradiation, and labelled with [³²P]orthophosphate. Cdk4 was then immunoprecipitated and separated by gel electrophoresis. It migrated as a band with a relative molecular mass (*M_r*) of 34K, as confirmed by western blot (Fig. 3b). Two-dimensional phosphoamino-acid analysis revealed that Cdk4 was phosphorylated on threonine but hardly at all on tyrosine (Fig. 3c). The content of phosphotyrosine, however, markedly increased in ultraviolet-induced G1-arrested cells. When the same analysis was done for Cdk4 from ultraviolet-irradiated M'13 cells, the content of phosphotyrosine markedly decreased despite there being a similar level of phosphothreonine, indicating that the phosphorylated tyrosine was Y17. Judging from its amount, the residual phosphotyrosine was

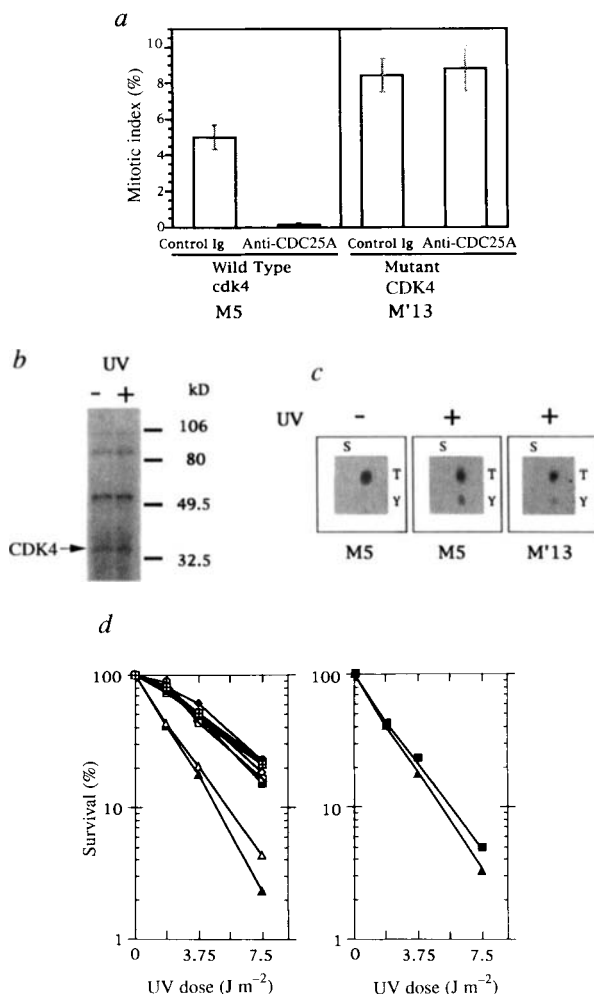


FIG. 3 *a*, Cells expressing Cdk4^{F17} do not require Cdc25A for cell cycle start. Synchronized M5 and M'13 were microinjected with the anti-Cdc25A antibody or control immunoglobulin. After 22 h incubation, cells undergoing mitosis were counted as a conventional assay for cell cycle 'start'. *b*, Cdk4 is phosphorylated in G1. M5 and M'13 were synchronized, UV-irradiated or mock-irradiated at 2 h and labelled from 6 to 12 h after stimulation with [³²P]orthophosphate. Cell lysates were then immunoprecipitated with an anti-Cdk4 antibody, electrophoresed on 12% SDS-polyacrylamide gels and autoradiographed. The results for mock-irradiated and UV-irradiated M5 are shown here. The numbers on the right indicate the *M_r* markers. *c*, Cdk4 is phosphorylated at tyrosine 17. The ³²P-labelled, immunoprecipitated, electrophoresed Cdk4 proteins from mock-irradiated and UV irradiated M5 and UV-irradiated M'13 were recovered from Immobilon membrane and acid hydro-

perhaps derived from phosphorylated Y17 of endogenous Cdk4 in M'13 (Fig. 1*c*). Taken together, these data indicate that Cdk4 was phosphorylated at Y17 during G1 and that the retention of the phosphorylated state was required for ultraviolet-induced G1-arrest.

Cells expressing Cdk4^{F17}, which are deficient in G1-checkpoint function, suffered highly elevated chromosomal aberrations and increased cell death. G1-synchronized cells, some of which were irradiated with ultraviolet, were incubated with colcemid, and resulting metaphase-blocked mitotic cell chromosomes analysed. Cells that were not irradiated were indistinguishable in the basal level of chromosome aberrations but, when ultraviolet-irradiated, only M'8 and M'13, the Cdk4^{F17} expressors, displayed a five- to tenfold increase in chromosomal aberrations (Table 1). Chromosome exchanges (large arrows) and chromatid-type breaks (small arrows) were particularly evident (Fig. 3*e*).

Cells deficient in G1-checkpoint function were more susceptible to ultraviolet, showing increased cell death (Fig. 3*d*). The

lysed. The hydrolysate was then separated by two-dimensional electrophoresis. The authentic phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) are indicated. *d*, Effect of UV irradiation at 2 h (left) or 12 h (right) on the survival of cell clones expressing an empty vector (H4, ○), Cdk2 (N3, ◆; N4, ◇), Cdk2^{F15} (N'16, □; N'19, ▤), Cdk4 (M2, ■; M5, ▨) or Cdk4^{F17} (M'8, ▲; M'13, △) and parental NRK (●). *e*, Two metaphase spreads prepared from M'13 cells irradiated with UV (7.5 J m⁻²) and cultured for 28 h. Chromatid type-breaks (small arrows) and exchanges (large arrows) are indicated. Scale bar, 30 μm. METHODS. *a*, Cell seeding, micro injection and analysis of cell cycling were performed as described⁴. *b*, M5 was synchronized, UV-irradiated or mock-irradiated at 2 h poststimulation, and labelled for 6 h before collection in phosphate-free DMEM containing 5% dialysed FCS and 1 mCi (37 MBq) [³²P]orthophosphate per ml. Cells were lysed with RIPA buffer containing 0.1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate. After centrifugation, supernatants were incubated for 3 h at 4 °C with the anti-Cdk4 antibody (C22). Protein G-Sepharose beads (Pharmacia) were added to the lysate and incubated for 1 h. Immunoprecipitates were collected by centrifugation, washed 5 times with RIPA buffer and 3 times with PBS at 4 °C, and electrophoresed on 12% SDS-polyacrylamide gels. *c*, Phosphoamino-acid contents of ³²P-labelled proteins were determined by partial hydrolysis of Immobilon membrane-bound proteins in constantly boiling 6 N HCl for 1 h at 110 °C (ref. 26). The resulting phosphoamino acids were separated by two-dimensional electrophoresis²⁷. *d*, G1-synchronized cells were plated at 100–1,000 cells per dish and irradiated with various doses of UV at 2 h (left) or 12 h (right) poststimulation. After a 14-day culture, all dishes were stained with Giemsa's solution. Only colonies containing more than 50 cells were scored as formed colonies. Three independent experiments were done with three dishes per each dose. *e*, See Table 1 for methods.

mean lethal dose for M'8 and M'13 was half of that for NRK cells. However, when irradiated at the G1/S boundary (12 h), both Cdk4 and Cdk4^{F17} expressors displayed the same level of chromosomal aberrations and cell death (Table 1; Fig. 3*d*), demonstrating that they were indistinguishable when irradiated after the execution point of G1 checkpoint arrest. Despite the elevated sensitivity to ultraviolet, their DNA repair function was normal. M'8 and M'13 had the same ability of unscheduled DNA synthesis as parental NRK cells (data not shown). We therefore conclude that their chromosome abnormalities arose from a lack of a G1 checkpoint function, not a DNA repair defect.

In fission yeast^{9,12} and mammals^{16,19}, Cdc2 is regulated by tyrosine phosphorylation and dephosphorylation, this regulation being a key mechanism controlling the onset of mitosis and G2 checkpoint arrest. Our results indicate that, in mammals, the same mechanism is used for ultraviolet-responsive G1 checkpoint control, but the target is Cdk4. Although yet to be demonstrated, tyrosine phosphorylation is likely to inactivate Cdk4

directly, as it does Cdc2 and Cdk2 (refs 13, 14), because the phosphorylation site is at the ATP binding domain which is conserved in all Cdk kinases. Strikingly, the cells in which G1 checkpoint function is no longer in operation as a result of Cdk^{F17} expression suffer elevated chromosomal breaks and exchanges with increased cell death upon ultraviolet irradiation. This type of aberration is typically seen in cells of the cancer-prone, G1 checkpoint-deficient genetic disorder ataxia telangiectasia^{20,21}. Our latest experiments suggest that tyrosine phosphorylation of Cdk4 is also involved in X-ray-induced G1 checkpoint arrest (unpublished data), indicating that tyrosine phosphorylation of Cdk4 (or maybe Cdk6 (ref. 22) for some other cells) is a common G1 arrest mechanism. □

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1. Xiong, Y. et al. *Nature* **366**, 701–704 (1993).
2. Zhan, Q., Carrier, F. & Fornace, A. J. Jr *Molec. cell. Biol.* **13**, 4242–4250 (1993).
3. Sherr, C. J. *Cell* **73**, 1059–1065 (1993).
4. Jinno, S. et al. *EMBO J.* **13**, 1549–1556 (1994).
5. Matsushima, H. et al. *Cell* **71**, 323–334 (1992).

6. Hoffman, I., Draetta, G. & Karsenti, E. *EMBO J.* **13**, 4302–4310 (1994).
7. Gould, K. L. & Nurse, P. *Nature* **342**, 39–45 (1989).
8. Parker, L. L. & Piwnicka-Worms, H. *Science* **257**, 1955–1957 (1992).
9. Enoch, T. & Nurse, P. *Cell* **60**, 665–673 (1990).
10. Lundgren, K. et al. *Cell* **64**, 1111–1122 (1991).
11. Rowley, R., Hudson, J. & Young, P. G. *Nature* **356**, 353–355 (1992).
12. Enoch, T., Carr, A. M. & Nurse, P. *Genes Dev.* **6**, 2035–2046 (1992).
13. Gu, Y., Rosenblatt, J. & Morgan, D. O. *EMBO J.* **11**, 3995–4005 (1992).
14. Sebastian, B., Kaizuka, A. & Hunter, T. *Proc. natn. Acad. Sci. U.S.A.* **90**, 3251–3524 (1993).
15. Ninomiya-Tsuji, J., Nomoto, S., Yasuda, H., Reed, S. I. & Matsumoto, K. *Proc. natn. Acad. Sci. U.S.A.* **88**, 9006–9010 (1991).
16. Heald, R., McLoughlin, M. & McKeon, F. *Cell* **74**, 1–20 (1993).
17. Igarashi, M., Nagata, A., Jinno, S., Suto, K. & Okayama, H. *Nature* **353**, 80–83 (1991).
18. Krek, W. & Nigg, E. A. *EMBO J.* **10**, 3331–3341 (1991).
19. Nagata, A., Igarashi, M., Jinno, S., Suto, K. & Okayama, H. *New Biol.* **3**, 959–968 (1991).
20. Nagasawa, H., Latt, S. A., Lalonde, M. E. & Little, J. B. *Mutat. Res.* **148**, 71–82 (1985).
21. Tatsuoka, M., Nikaide, O., Tatsumi, K. & Takebe, H. *Mutat. Res.* **214**, 321–328 (1989).
22. Meyerson, M. & Harlow, E. *Molec. cell. Biol.* **14**, 2077–2086 (1994).
23. Vindelov, L. L., Christensen, I. J. & Nissen, N. I. *Cytometry* **3**, 323–327 (1983).
24. Mizushima, S. & Nagata, S. *Nucleic Acids Res.* **18**, 5322–5326 (1990).
25. Chen, C. & Okayama, H. *Molec. cell. Biol.* **7**, 2745–2752 (1987).
26. Kamps, M. P. *Meth. Enzym.* **201**, 21–27 (1991).
27. Cooper, J. A., Sefton, B. & Hunter, T. *Meth. Enzym.* **99**, 387–402 (1983).

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Crystal structure of a replication fork single-stranded DNA binding protein (T4 gp32) complexed to DNA

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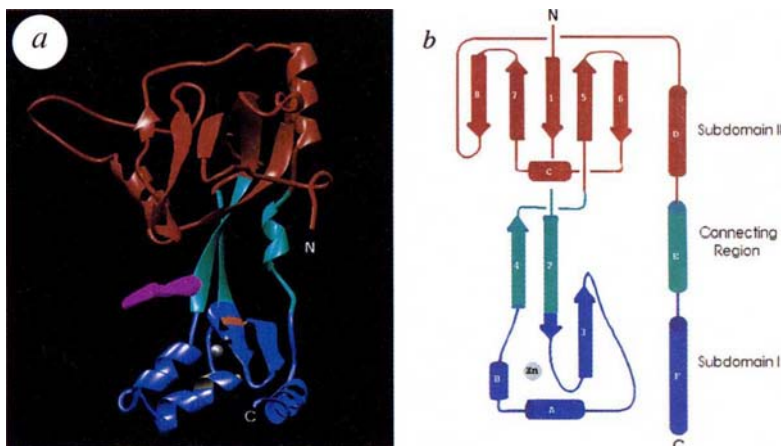
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THE single-stranded DNA (ssDNA) binding protein gp32 from bacteriophage T4 is essential for T4 DNA replication, recombination and repair. *In vivo* gp32 binds ssDNA as the replication fork advances and stimulates replisome processivity and accuracy by a factor of several hundred¹. Gp32 binding affects nearly every major aspect of DNA metabolism. Among its important functions are: (1) configuring ssDNA templates for efficient use by the replisome including DNA polymerase; (2) melting out adventitious secondary structures; (3) protecting exposed ssDNA from nucleases; and (4) facilitating homologous recombination by binding ssDNA during

strand displacement. We have determined the crystal structure of the gp32 DNA binding domain complexed to ssDNA at 2.2 Å resolution. The ssDNA binding cleft comprises regions from three structural subdomains and includes a positively charged surface that runs parallel to a series of hydrophobic pockets formed by clusters of aromatic side chains. Although only weak electron density is seen for the ssDNA, it indicates that the phosphate backbone contacts an electropositive cleft of the protein, placing the bases in contact with the hydrophobic pockets. The DNA mobility implied by the weak electron density may reflect the role of gp32 as a sequence-independent ssDNA chaperone allowing the largely unstructured ssDNA to slide freely through the cleft.

Since its isolation², gp32 has served as a prototype for a ubiquitous class of proteins collectively termed ssDNA binding proteins (SSBs)³. Other members of this class of essential DNA replication proteins include *Escherichia coli* SSB, T7 SSB 2.5, adeno-DBP and human/yeast RP-A¹. The idea that these proteins discriminate between single-stranded and double-stranded DNA via hydrophobic interactions, involving aromatic side chains between the protein and the DNA bases, has been strongly supported by numerous biochemical studies of gp32 (refs 3–6). Gp32 consists of 301 residues (*M*_r 33,488) which can be divided into three distinct domains by limited proteolysis¹. Removal of the amino-terminal 17 residues results in a complete loss of gp32:gp32 cooperativity. The C-terminal 46 residues are essential to the interactions of gp32 with other proteins, such as T4 DNA polymerase. Removal of both domains leaves 'core gp32' (residues 21 to 254), which has the same intrinsic affinity for ssDNA as the intact protein⁷.

FIG. 1 *a*, Ribbon diagram of core gp32 showing the subdomain structure and general positioning of the ssDNA made using the program RIBBONS²⁶. The protein fold of subdomain I (dark blue) is determined by the coordination about the Zn²⁺ (silver sphere). The position of zinc ligand His 64 is shown as an orange band, and Cys 77, Cys 87 and Cys 90 are yellow. Subdomain II (red) comprises five β-strands that form a twisted β-sheet which sits roughly perpendicular to the page. Part of the ssDNA binding cleft is made up of this sheet. The 'connecting' region (light blue) links subdomains I and II. All three regions contribute to making up the ssDNA binding cleft of the protein. The track of the phosphate backbone of the ssDNA is shown in pink. *b*, Topology map of the secondary structure of core gp32.



We have solved the crystal structure of core gp32 complexed with the oligonucleotide p(dT)₆ at 2.2 Å resolution (Fig. 1), using a combination of multiple isomorphous replacement (MIR) and multiwavelength anomalous diffraction (MAD) methods (Table 1, Fig. 2a, b). The structure (residues 22 to 239) of the ssDNA binding domain (Figs 1 and 2e) consists of three substructures: I, a Zn²⁺ binding subdomain; II, a 5-stranded β-sheet that is twisted about a central axis by about 30°; and a connecting region that joins these two.

The Zn²⁺ ion of subdomain I is tetrahedrally coordinated by His 64, Cys 77, Cys 87 and Cys 90 (Fig. 2a). Removal of Zn²⁺ from gp32 makes the protein hypersensitive to proteases and markedly decreases its thermostability, suggesting that the zinc ion coordination is important for conformational stability^{8,9}. Earlier attempts to identify the zinc ligands by analogy to zinc-finger proteins suggested the involvement of His 81, which gave a typical ligand spacing of Cys-X₃-His-X₉-Cys-X₂-Cys (residues 77 to 90) rather than the (His-X₁₂-Cys-X₉-Cys-X₂-Cys) (residues 64 to 90) observed here⁹. The structure shows that His 64 is a ligand to the Zn²⁺, and His 81 is positioned in a surface-exposed loop more than 12 Å away from the zinc ion. Two of the Zn²⁺ ligands (Cys 87 and Cys 90) are found in helix A (Fig. 1), whereas the other two (His 64 and Cys 77) are found in β-strands (Figs 2 and 3, respectively). Unlike zinc domains found in zinc-

fingers, such as TFIIIA, the two β-strands in gp32 are roughly perpendicular to the axis of the α-helix, rather than parallel to it.

Although the growth of this crystal form required the presence of dT₆ which must be phosphorylated ($K_a = 5 \times 10^6 \text{ M}^{-1}$), and reverse-phase HPLC analysis of the crystals indicated a stoichiometric amount of DNA (data not shown), we observed weak electron density for three nucleotides in the original solvent-flattened maps (Fig. 2c). Because of the weak electron density observed in our original maps, we used alternative oligonucleotides with a variety of sequences in an attempt to produce more interpretable maps. Cococrystallization of core gp32 with 5'-pTTATTT and 5'-pTTTATT consistently showed extra electron density in a deep cleft of the protein surface, a finding that could not be explained by the protein model.

We have fitted a tetranucleotide into the difference electron density of a map calculated using $2F_o - F_c$ as coefficients, and phases derived from a partly refined model, and the original experimental solvent-flattened maps (Fig. 2c, d). These maps contain electron density for Thy 1-4 which are largely in an unstacked and extended conformation (Fig. 2c). The ssDNA fits easily (Figs 1a and 3b, c) with the bases located in hydrophobic pockets lining the electropositive cleft. The overall DNA binding site size of core gp32 determined by fluorescence quenching experiments is 6 nucleotides^{4,10}. However, the intrinsic associa-

TABLE 1 Crystal structure statistics for gp32

(a) Native and MIR data collection statistics		Resolution (Å)	Measurements	Unique reflections	Completeness (%)	R_{sym} (%) [*]	R_{diff} (%) [†]	Phasing power [‡]
Native with pTTTTT		2.2	33,415	16,406	91.7	5.8		
pTTATTT		2.9	41,411	6,747	93.3	9.4		
pTTTATT		2.9	17,904	6,747	87.6	9.8		
trimethyl lead acetate		3.0	20,774	6,135	97.2	9.6	12.3	0.81
Se-met(E4) [§]		3.1	29,171	5,643	93.2	7.7	19.2	0.77
(b) MAD data collection statistics		Wavelength (Å)	Anomalous scattering factors f' f''	Measurements	Unique	Completeness (%)	R_{centric} (%) 25–5.4 Å	R_{overall} (%) 25–5.4 Å
E1	0.9960	–9.6	3.6	28,761	5,623	92.9	3.8	6.7
E2	0.9978	–6.4	4.4	28,821	5,627	93.0		
E3	0.9984	–5.7	4.2	28,748	5,613	92.8		
E4	1.0000	–3.9	3.8	29,171	5,643	93.2	5.0	9.3
(c) MAD and MIR phase determination statistics		Resolution range (Å)	$R(F(z))$ (%) [☆]	$R(F(a))$ (%) [☆]	f.o.m.-MAD #	f.o.m.-MIR #	f.o.m.-MAD + MIR # ^{**}	
25.0 6.3		5.4	34.3	0.81	0.77	0.94		
6.3 5.0		6.4	41.0	0.74	0.65	0.87		
5.0 4.4		6.9	45.9	0.67	0.52	0.81		
4.4 4.0		7.6	49.6	0.62	0.44	0.78		
4.0 3.7		9.1	50.9	0.46	0.24	0.65		
3.7 3.4		9.7	42.7	0.44	0.21	0.65		
3.4 3.1		12.4	37.3	0.35	0.19	0.58		
Overall 25.0 3.1		7.2	41.7	0.60	0.40	0.77		

Core gp32:p(dT)₆ complex was crystallized in 20% PEG8000, 100 mM ammonium sulphate, 50 mM sodium chloride, 5% ethylene glycol and 20 mM N-(2-hydroxyethyl)-piperazine-N'-(3-propane sulphonic acid), pH 8.4 (EPPS) in the space group P6₅22 with $a = 66.5 \text{ Å}$, $c = 235.4 \text{ Å}$. a, Native data were collected on the CHESS A1 beamline CCD detector. The lead derivative was prepared by soaking native crystals in 10 mM trimethyl lead acetate for 48 h before data collection on a Rigaku R-axis II Detector/CuK_α source. All data shown were collected at –170 °C, processed with the program DENZO, and scaled with SCALEPACK¹⁹. Heavy atom positions were determined by difference Patterson (CCP4)²⁰. Heavy atom phase refinement was done with the program ML-PHARE and combined with the MAD phase set to calculate an electron density map²¹. b, Seleno-methionine core gp32 was purified from *E. coli* MIC88 (a methionine auxotroph) containing plasmid pYS55 induced in defined media²². Multiwavelength data were collected on the CHESS F2 beamline using image plates and a Kodak Scanner. X-ray fluorescence spectra of the crystals were used to optimize the data collection strategy and included complete reverse beam to measure Bijvoet pairs. c, MAD phases were calculated using the program MADPRB (A.M.F., unpublished). Over the entire resolution range (25 to 3.1 Å), 77% of the unique reflections were phased by MAD. The combined MAD/MIR phase set produced clearly interpretable electron density that was subsequently solvent flattened using SQUASH²³ and the model built in O (ref. 24). The current model includes residues 22 to 239, and has been partly refined against the native data from 8.0 to 2.2 Å in X-PLOR²⁵. Without the inclusion of ssDNA, but with 48 water molecules, the model has an R-factor of 24.1% and an r.m.s. for bond lengths of 0.011 Å and for bond angles of 0.016°. Two non-glycine residues lie outside the allowed regions of a Ramachandran plot and they are located at the surface.

^{*} R_{sym} is the unweighted R-factor on intensities for multiple observations of symmetry related reflections.

[†] R_{diff} is the unweighted R-factor for the differences between native (N) and derivative (D) data expressed as $|F_D - F_N|/F_N$.

[‡] Phasing power is defined as r.m.s. $F_{\text{HA}}/r.m.s.$ residual.

[§] The remote E4 wavelength was used as the data set for calculation of isomorphous differences between seleno-methionine and methionine core gp32. E4 was chosen because f'' is the least negative at this wavelength and consequently gives the greatest isomorphous differences.

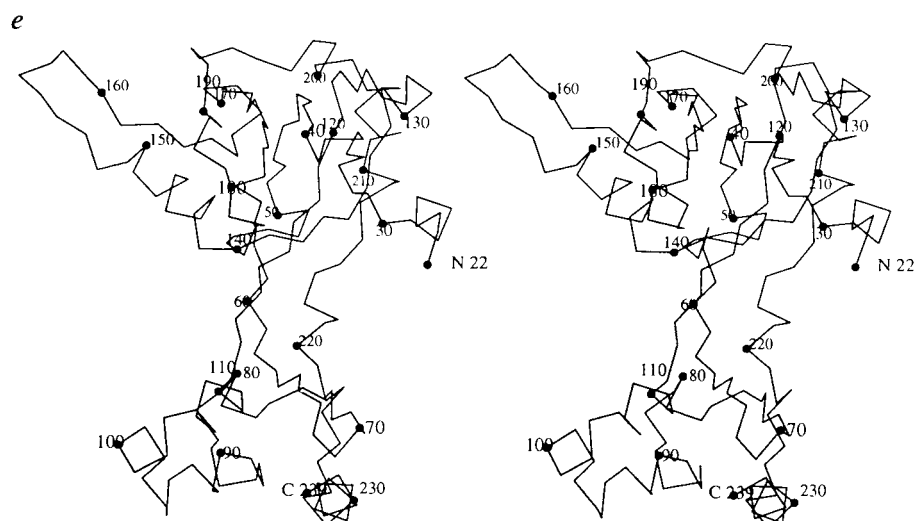
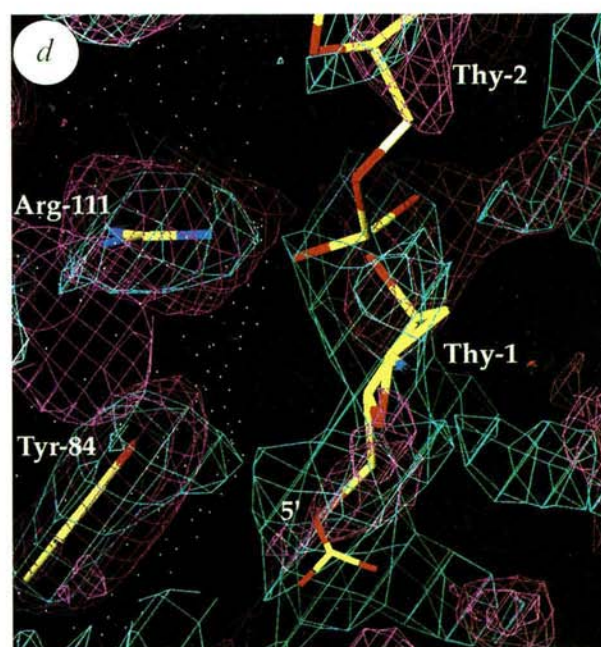
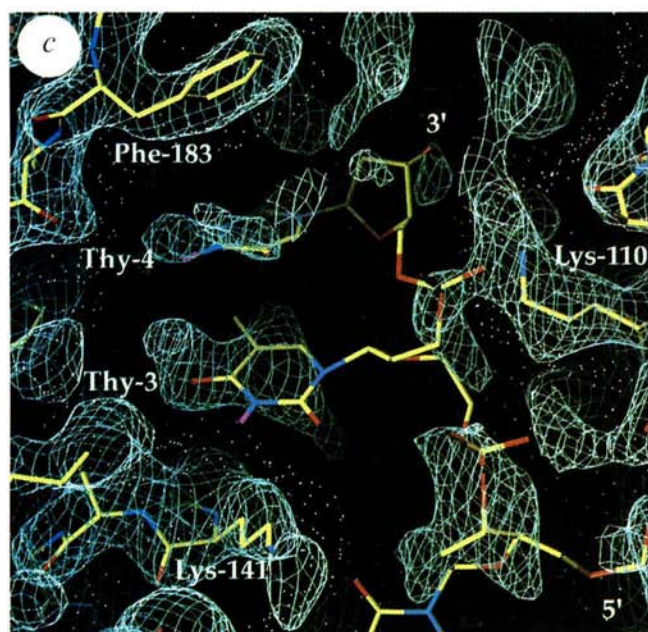
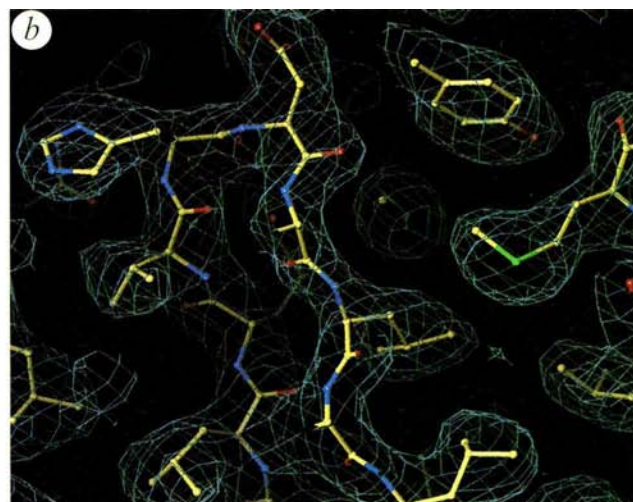
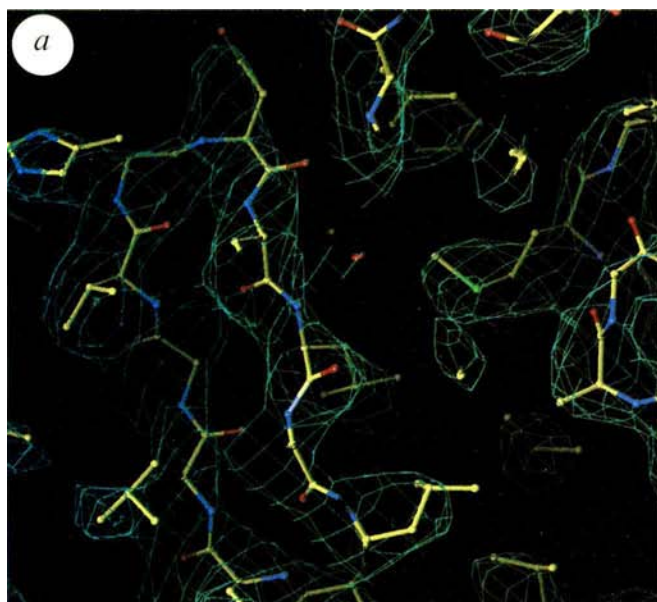
^{||} The remote wavelengths f' and f'' were held fixed at theoretical values during refinement.

[¶] R_{centric} is the R-factor on intensity for the centric data. In the case of perfect data, the $R_{\text{centric}} = 0\%$. In this case, R_{centric} is a good indicator of experimental error. When compared with R_{overall} , which treats all the dispersive and Bijvoet differences as equivalent, a general measure of the MAD signal is obtained.

[#] f.o.m. is figure of merit, defined as $\cos \langle \sigma(\Delta\phi) \rangle$.

[☆] Where $R = \sum_i \sum_j |F_i(h) - \langle F(h) \rangle| / \sum_i \sum_j F_i(h)$, the summation taken over redundant phase determinations for either the wavelength invariant structure factor $F(z)$, or the anomalous structure factor $F(a)$.

^{**} Combined f.o.m. includes reflections for which both MAD and MIR measurements were available.



◀ FIG. 2 *a*, Experimental electron density map to 3.1 Å contoured at 2.5 σ and calculated using the combined MAD and MIR phases that have been solvent flattened. The coordination of the Zn²⁺ ion (yellow) is tetrahedral with His 64, Cys 77, Cys 87 and Cys 90 as ligands. *b*, 2F_o - F_c electron density map contoured at 1.3 σ showing a stretch of β -strand 4 that includes the current partly refined model and all the data to 2.2 Å. *c*, 2F_o - F_c electron density map to 2.2 Å of Thy 2 to Thy 4. The maps were made using SIGMAA weighted phases²⁰ from the partly

refined structure that does not include the DNA model. The map is contoured at 0.8 σ . The molecular surface of the protein is indicated with white dots as illustrated in Fig. 3a. *d*, Electron density maps of the 5' end of the hexanucleotide calculated with 2F_o - F_c amplitudes as in *c* (pink) or with F_o's from core gp32:ptTATT cocrystals to 2.9 Å (blue). Maps are contoured at 0.8 σ . *e*, Stereo α -carbon backbone structure of core gp32 residues 22 to 239 (made using the program MOLSCRIPT²⁷).

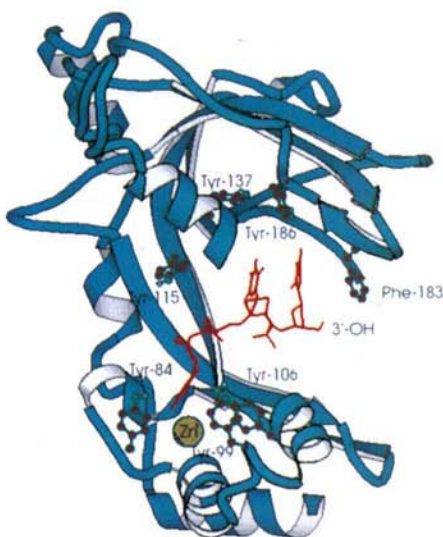
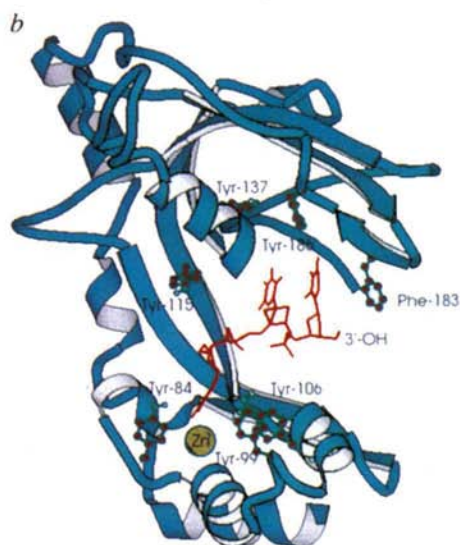
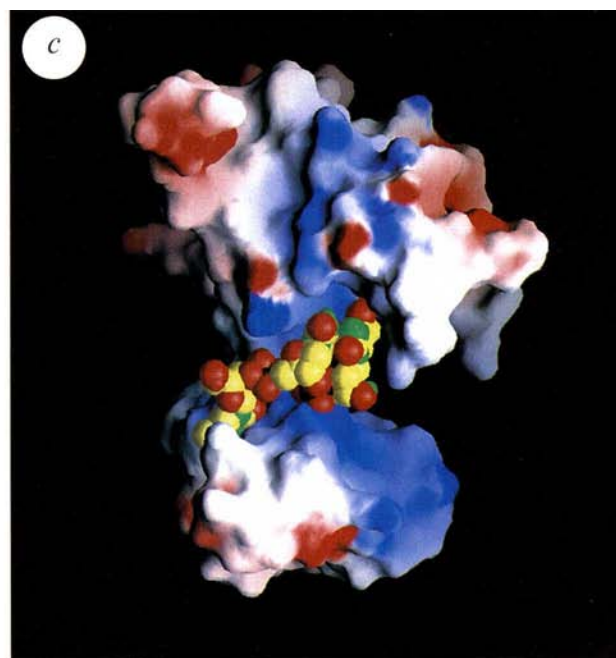
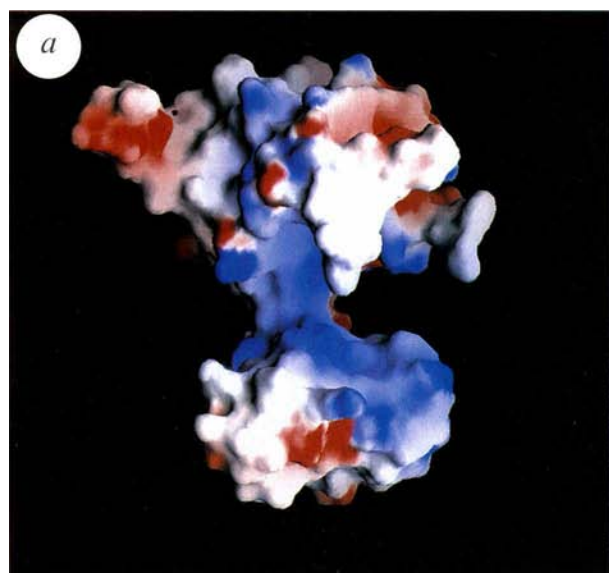


FIG. 3 *a*, Representation of the electrostatic surface potential of core gp32 (made using GRASP²⁸). The orientation of the surface is the same as in Fig. 1a, with the ssDNA binding cleft running from left to right through the strongly electropositive (blue) region of the protein (electronegative regions are displayed in red). Residues contributing to this electropositive cleft are Lys 110, Arg 111, Lys 112, Lys 136, Arg 138 and Lys 141. It has been suggested²⁹ that, when ssDNA is not bound, residues 110 to 114 interact with a negatively charged region. When binding occurs, the negatively charged region is displaced by ssDNA and may then interact with a positive region of an adjoining monomer to nucleate cooperative binding²⁹. A stretch of basic residues Lys-Arg-Lys-Ser-Thr (residues 110 to 114) is identically repeated in the N terminus (residues 3 to 7) of the intact protein, and is known to be essential

for gp32:gp32 cooperativity. *b*, Stereo view of core gp32 showing those residues shown by site-directed mutagenesis⁴ (at positions Tyr 84, 99, 106, 115, 137, 186) or photochemical crosslinking¹³ (Phe 183) to be involved in ssDNA binding. Mutation of these tyrosines results in decreases in the intrinsic affinity for ssDNA or its ability to differentiate ss- from dsDNA⁴. The phosphate backbone of the ssDNA is shown as a stick representation. *c*, The ssDNA (carbon, yellow; oxygen, red; nitrogen, green; phosphate, black) on the electrostatic surface potential diagram of core gp32. The ability of gp32 to differentiate ss versus dsDNA is based on limiting the binding groove to a rather narrow 15 Å dimension in the middle of the cleft formed by the loop containing Phe 183 and Tyr 186.

tion constant to oligonucleotides remains constant once a minimum size of three nucleotides is reached, suggesting tight binding between gp32 and 2–3 nucleotides¹⁰, an observation consistent with the crystal structure. The polarity of the tetranucleotide cannot be unambiguously assigned from the electron density maps, but has been fitted in the cleft between Tyr 115/Trp 144 and Tyr 84 (Fig. 2d). This is consistent with ¹H-NMR studies that have shown Tyr 115 to be one of the residues affected when core gp32 binds short oligonucleotides^{5,11}, as well as with other studies that have suggested the involvement of one tryptophan residue (presumably Trp 144) in binding^{10,12}. Thy 5 and Thy 6 are disordered but would presumably be found at the other end of the cleft, near Trp 72. The phosphate oxygens of Thy 2 make an electrostatic interaction with Arg 111 (Fig. 2d). There appears to be some rotational and translational freedom about the phosphodiester backbone which would allow the base to be fairly mobile within the cleft. Such freedom of motion by the ssDNA bases might help explain the weak electron density observed for the DNA in this complex.

The base of Thy 3 appears to make an end-on interaction with Tyr 186 and stack with the base of Thy 4 (Fig. 2c). Thy 4 has the potential to stack with the base of Thy 3 and Phe 183 (4–5 Å), and its 3' phosphate oxygens make electrostatic contacts with Lys 110. Phe 183 can be photochemically crosslinked to p(dT₈), suggesting that it lies very near one of the bases¹³, and in our model Phe 183 makes stacking interactions with Thy 4 (Fig. 3b). Phe 183 is part of a solvent-exposed loop that protrudes into the ssDNA binding cleft of the protein, narrowing it such that double-stranded DNA cannot fit.

Although core gp32 bears little resemblance to the RNA binding domains of small nuclear ribonucleoprotein U1A¹⁴, the ssDNA packaging protein gp5 (ref. 15) from phage ϕ d or adenovirus DNA binding protein¹⁶, the structure of the core gp32:ssDNA complex illustrates a recurrent feature in all the known single-stranded nucleic acid binding proteins. All four proteins use aromatic side chains from proximal β -strands to form pockets for the recognition of bases. Data from electron microscopy¹⁷ and quasi-elastic light scattering¹⁸ have shown that gp32 extends the distance between bases from a standard B-DNA distance of 3.4 Å to between 4.3 and 5.4 Å, in good agreement with our own estimate of 5 Å. This results in unstacking of the bases, which has been observed experimentally for poly ribo-ethanoadenylic acid bound to gp32 (ref. 1).

Although ssDNA disorder in these crystals could be due to some feature of the crystal environment, it seems more likely that it reflects the role of gp32 as a replication fork ssDNA binding protein. Disordered ssDNA results from the binding of ssDNA in a variety of conformers that are relatively isoenergetic with respect to each other. Gp32 is presented with the seemingly paradoxical problem of binding ssDNA tightly enough to pre-

clude the formation of secondary structures, yet allowing the DNA to be accessed by T4 DNA polymerase as well as other proteins of T4 recombination, replication and repair. The ssDNA disorder may reflect an energy barrier to ssDNA translocation that is small compared with thermal energy (kT). Such a small energy barrier would make movement of ssDNA through the gp32 cleft into the active site of the DNA polymerase during translocation possible without requiring gp32 dissociation. During replication, gp32 must bind ssDNA of varying sequences with roughly equal affinity. To do this, gp32 affinity must be based on the common characteristics of nucleotides, such as hydrophobic interactions between the bases and aromatic side chains, as well as electrostatic interactions between phosphate oxygens and lysine and arginine residues, rather than sequence-specific hydrogen-bonding networks. We do not observe intercalation of aromatic side chains into ssDNA, but rather the positioning of bases in pockets lined with aromatic side chains (Fig. 3b). This is consistent with NMR studies showing only small ring current shifts between core gp32 and bound oligonucleotides^{3,5}. Although gp32 does not strictly limit DNA conformations to the extent seen in repressor:operator interactions, it does restrict free rotation about the phosphodiester backbone, and prevents the formation of intramolecular secondary structures that would impede translocation by the polymerase.

Although gp32 strongly stimulates renaturation of T4 DNA², the structure of the core gp32:ssDNA complex makes it unlikely that two strands of ssDNA coated by gp32 could renature without first dissociating. Severe steric clashes occur when attempts are made to dock a second strand onto the bound DNA. This is in excellent agreement with studies indicating that gp32 has a 10,000-fold lower affinity for double-stranded DNA (dsDNA) and is unable to lower the melting temperature or denature T4 dsDNA¹. If gp32 could simultaneously bind ssDNA and renature duplex DNA, the replication fork would be re-annealed by gp32 rather than being kept open. Rather, we suggest that gp32 may stimulate reannealing of DNA simply by removing secondary structures and extending the DNA. As gp32 translocates or ssDNA transiently dissociates, nucleation of renaturation can begin, followed by a rapid zippering up of the ssDNA into dsDNA that pushes gp32 ahead of the advancing duplex, or dissociates gp32 from the single strand. We believe that this translocation or sliding of gp32 during reannealing is essentially identical to the process that gp32 undergoes in front of the rapidly advancing replisome. As the T4 DNA polymerase advances, gp32 may slide, allowing the ssDNA to move through its cleft into the polymerase. By its ability to configure ssDNA into an irregular extended helix devoid of secondary structures, and protecting it from nucleases, gp32 fulfils the role of a ssDNA chaperone by delivering the unstructured ssDNA essential to T4 replication, recombination and repair. □

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- Karpel, R. L. in *The Biology of Nonspecific DNA-Protein Interactions* (ed. Revzin, A.) 103–126 (CRC, Boca Raton, FL, 1990).
- Alberts, B. L. & Frey, L. *Nature* **227**, 1313–1318 (1970).
- Prigodich, R. V., Casas-Finet, J., Williams, K. R., Konigsberg, W. H. & Coleman, J. E. *Biochemistry* **23**, 522–529 (1984).
- Shamoo, Y. et al. *Biochemistry* **28**, 7409–7417 (1989).
- Pan, T., King, G. C. & Coleman, J. E. *Biochemistry* **28**, 8833–8839 (1989).
- Anderson, R. A. & Coleman, J. E. *Biochemistry* **14**, 5485–5491 (1975).
- Spicer, E. K., Williams, K. R. & Konigsberg, W. H. *J. Biol. Chem.* **254**, 6433–6436 (1979).
- Giedroc, D. P., Keating, K. M., Williams, K. R. & Coleman, J. E. *Biochemistry* **26**, 5251–5259 (1987).
- Giedroc, D. P., Johnson, B. A., Armitage, I. M. & Coleman, J. E. *Biochemistry* **28**, 2410–2418 (1989).
- Kowalczykowski, S. C., Lonberg, N., Newport, J. W. & von Hippel, P. J. *Molec. Biol.* **145**, 75–104 (1981).
- Prigodich, R. V. et al. *Biochemistry* **25**, 3666–3672 (1986).
- Khamis, M. I. & Maki, A. H. *Biochemistry* **25**, 5865–5872 (1986).
- Shamoo, Y., Williams, K. R. & Konigsberg, W. H. *Protein Struct. Funct. Genet.* **4**, 1–6 (1988).
- Nagai, K., Oubridge, C., Jessen, T. H., Li, J. & Evans, P. R. *Nature* **348**, 515–520 (1990).
- Skinner, M. M. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 2071–2075 (1994).
- Tucker, P. A. et al. *EMBO J.* **13**, 2994–3002 (1994).
- Delius, H., Mantell, N. J. & Alberts, B. J. *Molec. Biol.* **67**, 341–350 (1972).

- van Amerongen, H., Kuil, M. E., Scheerhagen, M. A. & van Grondelle, R. *Biochemistry* **29**, 5619–5625 (1990).
- Otinowski, Z. in *Data Collection and Processing* (eds Sawyer, L., Isaacs, N. & Bailey, S.) 56–62 (SERC Daresbury Laboratory, Warrington, 1993).
- Collaborative Computational Project, Number 4 SERC Daresbury Laboratory, Warrington, UK *Acta crystallogr.* **D50**, 760–763 (1994).
- Otinowski, Z. in *Isomorphous Replacement and Anomalous Scattering* (eds Wolf, W., Evans, P. R. & Leslie, A. G. W.) 50–55 (SERC Daresbury Laboratory, Warrington, 1991).
- Yang, W., Hendrickson, W. A., Kalman, E. T. & Crouch, R. J. *J. Biol. Chem.* **265**, 13553–13559 (1990).
- Zhang, K. Y. J. *Acta crystallogr.* **D49**, 213–222 (1993).
- Jones, T. A., Zou, J.-Y., Cowan, S. W. & Kjeldgaard, M. *Acta crystallogr.* **A47**, 110–119 (1991).
- Brunker, A. T. X-PLOR (Version 2.2). Howard Hughes Medical Institute and Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut.
- Carson, M. J. *Appl. Crystallogr.* **24**, 958–961 (1991).
- Kraulis, P. J. *Appl. Crystallogr.* **24**, 946–950 (1990).
- Nicholls, A., Sharp, K. & Honig, B. *Proteins* **11**, 281–285 (1991).
- Casas-Finet, J., Fischer, K. R. & Karpel, R. L. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1050–1054 (1992).

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Tools of the trade

Tunable optical parametric generators, an expanded range of high-temperature tube furnaces, a new class of fibre optic instruments and a protein fingerprinting system are but a few examples of new equipment available for the laboratory.

Optilas has released the OPG902 series of **ultrashort pulse, picosecond, tunable optical parametric generators (OPG)** from BM-Industries (*Reader Service 100*). For applications in nonlinear-optical and ultrafast-spectroscopic studies, these lasers introduce all solid-state tuning from the UV to mid-IR wavelengths (410 nm to 2.6 μm), with pulse durations of 30ps and output energies up to 1.5 mJ. The OPG902 laser is pumped at either 355 nm or 532 nm using the second or third harmonic of a BMI 500DPS mode-locked Nd:YAG or Nd:YLF laser. The OPG902 can also be adapted by BMI to other commercial pump lasers. Three models of the OPG902 are in production, including a model that employs a special grating-based design that provides for a narrow line-width emissions. The whole tuning range is achieved without any optics change, and is controlled from a remote keypad or a personal computer.

Carbolite has expanded its range of **high-temperature tube furnaces** to include models with maximum operating temperatures of 1,500 °C, 1,600 °C, 1,700 °C and 1,800 °C (*Reader Service 101*). The 1,500 °C and 1,600 °C models accept work tubes up to 75 mm internal diameter and provide heated lengths of 450 mm and 610 mm. The equipment can

mm and 600 mm. Four vertical PVT furnaces incorporate lanthanum chromite elements to achieve a maximum operating temperature of 1,800 °C. Maximum work tube dimensions are 900 mm long $\times$ 125 mm internal diameter. Heated lengths are either 200 mm or 350 mm.

JDS Fitel has announced the availability of a new class of **fibre optic instruments** capable of introducing a variable delay into a light path (*Reader Service 102*). The HD3/HD4 series instruments introduce delays of up to 350 picoseconds and can be used in the polarization mode for dispersion-interferometric measurements. The instruments are also ideal for controlling the phase during the testing of reflection effects on detectors. The resolution is 0.002 ps (nominal) with a repeatability of ± 0.02 ps while return loss is >45 dB and insertion loss typically <3 dB. The instruments may be supplied optionally with polarisation preserving fibre and operate broadband over the range from 1,250 nm to 1,700-nm.

The **acoustic doppler velocimeter (ADV)**, developed by SonTek and available from Wallingford, is designed to be a high precision instrument for measuring all three flow velocity components of water in the laboratory (*Reader Service 103*). It uses acoustic sensing techniques, which means that the sampling volume is not disturbed by the presence of the probe. With a sampling volume of less than 0.25 cm^3 and a sampling rate of up to 25 Hz this probe offers an order of magnitude improvement over traditional sensing techniques. This small, robust probe is simple to use and gives consistent readings over a wide range of applications. The ability of the ADV to record velocities up to $\pm 2.5 \text{ m s}^{-1}$ with high resolution makes it a useful tool in the teaching of hydraulics, hydrodynamics and oceanography. It is ideal for use with a range of models in the laboratory from tabletop circulation flumes to large wave basins. The acoustic sensor consists of a transmitting transducer surrounded by three receiving transducers mounted on short arms at 120° intervals. The beams are orientated so that the sampling volume is located either at 50 mm or 100 mm below the probe. This geometry ensures that the flow within the sampling volume is unaffected by the presence of the probe itself.

Oxford Instruments' Optistat CF is a **general purpose, flexible optical cryostat**



Oxford Instrument's Optistat CF.

designed for use in applications such as spectroscopy, where optical stimulation of the sample is required (*Reader Service 104*). The optical benefits of this cryostat are its access ($f/1$) for light collection measurements, a 15 mm-clear beam throughput allowing a large illumination area for small signal measurements and the availability of a wide range of window materials covering most wavelengths. According to the manufacturer the cryogenic benefits include a base temperature 1.6 K with continuous operation and an optimised thermodynamic design, which allows minimal temperature differences between the heat exchange and sample holder, even while changing temperatures. Helium consumption has also been reduced by about 25 per cent.

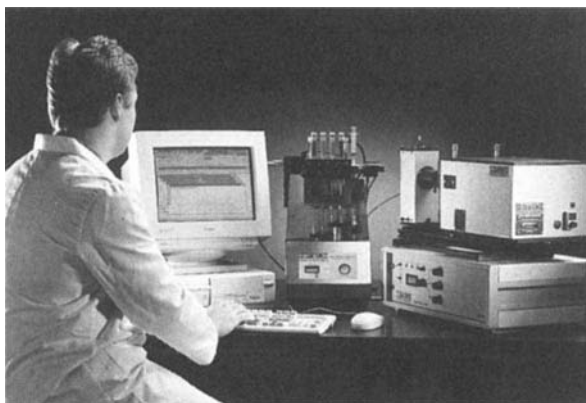
The PW2400 from Philip is an **elemental analyser** for industrial or research applications (*Reader Service 105*). The system features ceramic-based super-sharp tube (SST) excitation, ultra-close optical coupling, a unique detector configuration, DOPS goniometer control and the latest Philips software which streamlines results, calculations and decision making. Several special-purpose variants of the SST are now available, such as the ultra-light element tube, which is fine-tuned for maximum excitation efficiency in the lower atomic number region. In addition, Sc, Cr and Mo anode models can be used for applications where the standard Rh anode is less desirable. This model's light-element detection is also improved by the availability of new synthetic multi-layer



Carbolite's 1500° C vertical tube furnace.

be adapted easily for vertical or horizontal use. Three-zone models are available for very uniform temperature control. The 1,700 °C models accept work tubes 650 mm and 950 mm long by 75 mm maximum internal diameter. Heated lengths are 300

monochromators for Be, B and N. The system is design to enable optical modules to be exchanged in under 60s, and the ceramic-insulated X-ray tube can be quickly rotated between the line- and point-focus positions, depending on application requirements. The system features a horizontal goniometer and all-purpose cradle with open access that accepts samples as large as complete 23-cm wafers, or objects weighing up to 500 g.



High-Tech Scientific's SF-61 DX-2.

Varian Associates has announced the availability of **mass spectrometry technology** that is designed to allow researcher to confirm with reliability the identity of compounds, even in complex matrices (*Reader Service 106*). This new technology, selected ion storage (SIS), is designed to generate unique waveforms that selectively trap the ions of the analytes to be identified, and reject other ions from the background matrix. Enough different ions can be stored from the analyte to allow a search of library databases in the clinical, pharmaceutical, and environmental industries. The combination of background matrix rejection, increased sensitivity for analytes of interest and confirmation of identity, is said to make SIS a powerful analytical tool.

Kinetics

A new computer-controlled, stopped-flow system has been developed by Hi-Tech Scientific for rapid **kinetics measurements** (*Reader Service 107*). The SF-61 DX-2 is a fully integrated instrument featuring a completely automated sample handling unit that can be configured for single or multiple-mixing applications. The KinetA-syst control package features comprehensive menus to guide the user through the system. Equally suitable for absorbance

and fluorescence measurements, even into the deep UV part of the spectrum, Multi-Shot measurements can be used both in single- and dual-mixing applications. Multiple-wavelength measurements can be made from a single shot using diode array techniques. Fluorescence polarization and anisotropy measurements are also available, and a conductivity detector allows the study of fast conductimetric reactions. Other options include a dual beam sampling facility, a standard reference channel, which allows small signals on a large background to be followed and also enables the system to be used as a standard spectrophotometer.

Sapidyne Instruments has introduced the KinExA model 1, which is capable of **measuring the true binding kinetics of unmodified biomolecules** (*Reader Service 108*). The manufacturer states that this is a step forward for this technology, as it does not require immobilization of one component on a solid phase, or limited to measuring labelled partners. Because this instrument measures kinetic rate constants in solution, there is no interference from the modified biomolecule or ligand due to surface attachment chemistry. In addition, spurious results arising from diffusion to the solid phase species are eliminated. It is also said to readily handle

whole cells, allowing measurement in biologically significant matrices.

Bioanalysis

Pierce has just released a 20-page **signal transduction brochure**, focusing on the new line of colorimetric protein kinase assay kits (*Reader Service 109*). With technical and ordering information, as well as performance data, the brochure provides a comprehensive guide to the company's selection of colorimetric assay kits for protein kinase C, protein kinase A, protein tyrosine kinase and a variety of accessory products. Also featured are the substrate choices and kit format options exclusive to the colorimetric assay kits.

The Protein Fingerprinting system from Promega can be used to generate a **detailed pattern of peptide fragments**, which is characteristic for the target protein and the protease used for cleavage (*Reader Service 110*). The system employs partial proteolytic cleavage of target proteins, which may be purified proteins in solution or, often more conveniently, gel slices excised from an SDS gel separation of a heterogeneous sample. A total of three proteases (endoproteinase Glu-C, endoproteinase Lys-C and alkaline protease) are supplied with the system providing additional power to discriminate between closely related proteins. Among other applications this system can be used to identify significant structural elements between proteins or, in a partially purified protein mixture, to determine if two distinct bands are structurally related, possibly as modified and unmodified polypeptides.

Rapid solution exchange in a perfusion bath, around the extremity of a patch pipette or whole cells, is now possible using the Bio-Logic RSC-100 rapid solution changer (*Reader Service 111*). Available from Intracel, the system comprises a microprocessor control unit and a precision acrylic head, mounted on a stepping motor drive. Experimental solutions to be assayed are contained in standard plastic syringes and fed by gravity to the head, which can have between nine and 36 injector tubes, depending on the model chosen. Very precise rotation of the head exposes the patch pipette or cell to the flow of a chosen tube. The tube size is small enough to avoid cross-contamination with adjacent jets, but large enough to perfuse most cell or patch sizes at low flow rates. The device achieves changes in the order of milliseconds between outlets. It can be operated manually, programmed from a PC or Macintosh computer via the standard RS232 interface, or triggered by a pulse generator.

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tion of recombinant protein A levels in buffer, dilute serum or cell culture supernatant (in the presence or absence of IgG) (*Reader Service 112*). Purified chicken antibody to protein A is used to precoat a 96-well plate to capture protein A in the sample. Detachable, coated wells provide flexibility and consistency for simultaneous runs. Prediluted, lyophilized standards are made from recombinant protein A. Assay results are determined in less than three hours and only 100 μ l of sample is required. The company states that this EIA is optimized with a sensitivity of <1 pg ml $^{-1}$ in buffer and 13.6 pg ml $^{-1}$ in IgG diluent. Each kit contains stable reagents sufficient for 96 determinations.

DNA sequences can now be read directly from an X-ray film into a computer without the need for a keyboard (*Reader Service 113*). The new Readermouse from Integra Biosciences can communicate directly with your computer. This special version is suitable for use with Windows and Macintosh systems and enables DNA sequences to be edited, checked, processed, stored and exported.

Electrophoresis

Bio-Rad offers CE Solutions a new publication that features complete descriptions of Bio-Rad's **products for capillary electrophoresis (CE)** (*Reader Service 114*). The complete CE product line includes the BioFocus family of CE instruments, application kits, buffers, reagents, standards and capillaries. A free copy of this brochure is available from the company.

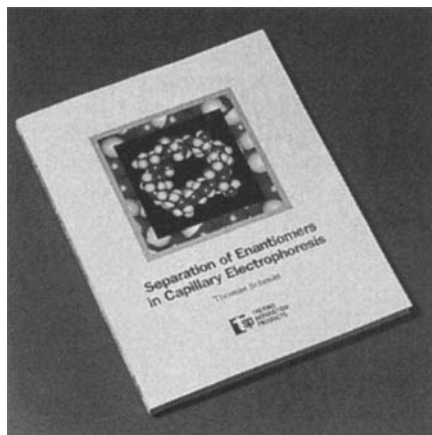
FMC states that Long Ranger **sequencing gels** engineered to produce sharp, clear, easy-to-read bands with significantly longer reads than polyacrylamide gels (*Reader Service 115*). Runs are said to be 50 per cent faster and to give up to 30 per cent more sequencing information than can be obtained in other sequencing techniques. The formulation of the gels is designed to eliminate the need for urea removal — minimizing handling, saving time and money. Also available are Pre-Mix formulations that have the added advantage of improved gel-to-gel reproducibility and ease of use.

The Nest Group offers **pre-cast mini gels** for native and denatured polyacrylamide gel electrophoresis (PAGE) of proteins, made through a new automated process, which is designed to give consistent gradients and high-resolution results (*Reader Service 116*). The manufacturer states that unlike pre-cast gels using Laemmli chemistry, these gel kits are guaranteed to last for up to a year. They use Tris-tricine chemistry for better protein fractionation of all molecular weights, especially low molecular-weight proteins. For added convenience, kits include pre-formed wells

with combs in place, 10 $\times$ running buffer mix, and SX sample buffer solution.

Savant's new digital **gel drying systems** offers enhanced controls for reproducible drying of all gel types (*Reader Service 117*). The self-contained SpeedGel (SG210D) is a complete system that occupies <45 cm of bench space. Featuring the integral, oil-free Gel Pump and recovery vessel for collection/disposal of condensed solvents, SpeedGel is designed to require no maintenance. Large sequencing gels or multiple smaller gels can be dried on the large, 40 cm $\times$ 50 cm drying surface. An LED display indicates temperature (30 $^{\circ}$ C–80 $^{\circ}$ C) during run and cool-down, or elapsed/remaining time. "Hold" mode provides extended drying >5 h. Savant also offers digital Slab Gel dryer (SGD2000) and oil-free Gel Pump (GP110), which combine to form a complete maintenance-free component system (GDS100).

A comprehensive overview of **methodologies for enantiomeric separations by capillary electrophoresis** has been pub-



..TSP's CE Separations Handbook.

lished by Thermo Separation Products (TSP) (*Reader Service 118*). The book reviews existing literature as well as describing methodologies.

Beckman's new **eCAP chiral methods development kit** contains everything needed for the rapid development of separation methods for optical isomers on the P/ACE capillary electrophoresis system (*Reader Service 119*). The chiral kit is designed to allow optimization of methods in days rather than weeks. Using the methods development scheme contained in the kit, resolution and selectivity can be maximized in as few as eight runs. Coupled with the automation features of the P/ACE system, unattended optimization is rapidly achieved by varying the concentration of cyclodextrin, pH and field strength. The eCAP kit contains two neutral capillaries, β -cyclodextrin, γ -cyclodextrin, hydroxypropyl- β -cyclodextrin dimethyl- β -cyclodextrin, three buffers, two pH adjusters, test

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mix, a guide and a diskette.

Bio Image has announced that its Intelligent Quantifier **electrophoresis image-analysis software** is now available as a multi-platform program for Windows 3.1, Macintosh and Sun systems (*Reader Service 120*). This software is a self-installing, self-tutoring program for analysing scanned images of one- and two-dimensional electrophoresis separations. The software automatically identifies lanes, then finds, quantifies, and sizes bands or spots. It quantifies blot cells and calculates unknown values from known concentrations; it finds and counts colonies and plaques. It is network ready and will import images from multiple-sources and can export data to multiple destinations. Users can annotate images for publication and automatically generate reports in formats suitable for presentation or export into popular spreadsheet and database programs.

Automation

Anachem now offers Gilson's Asted XL, an **on-line system for preparing and injecting HPLC samples** (*Reader Service 121*). Complex matrices, such as serum, plasma, urine, milk, meat and plants can be separated prior to on-line HPLC analysis. The entire process is fully automated and can take as little as 3 min to run. The apparatus has been applied to the measurement of levels of drugs, amino acids, catecholamines, steroids, vitamins, sugars and offers flexibility to develop a wide variety of applications. A guide and software have been produced to assist in early method development and a technical brochure is also available. The system is designed to run efficiently with any HPLC system.

Chromacol has launched a new line of **autosampler vials**, the New 2-CV (*Reader Service No. 122*). These vials have been designed to fit the majority of autosamplers on the market, and has two new features: a ceramic 'write on' label, to allow sample identification without the risk of a label fouling the autosampling sequence, and a wider opening, which is used to minimize damage to wayward autosampler needles. The larger opening to the vial has means that the low-volume glass inserts (220 µl capacity) now have a wider insert, so that the target area of the vial and the insert have been considerably increased.

Rheology

TA Instruments now offer a complete **rheological-system software** for the Windows systems, designed for the rheological characterization of liquids and semisolids (*Reader Service 123*). The instrument features automatic gap setting, inertia correction, air-bearing friction correction and controlled rate capability. The new soft-

ware packages offer flow, creep, oscillation and data analysis in the Windows operating system. The software provides complete instrument control and an analysis package with data calculated in comparison to a comprehensive list of the theoretical models. The operating environment allows the operator to multi-task and to easily export data to other Windows software packages and a variety of output devices, including laser printers, plotters, clipboards, tape drives and networks.

Miscellaneous

J. T. Baker now offers the SAF-T Disc program, with **Material Safety Data Sheets (MSDSs) on CD-ROM** (*Reader Service 124*). The SAF-T Disc program is purchased as a subscription with quarterly updates. The single disc contains over 1,800 J. T. Baker data sheets in English, Spanish and French. In addition to being network compatible, the SAF-T disc CD-ROM program will run on DOS, Windows and Macintosh systems. Material Safety Data Sheets may be viewed or printed utilizing the search criteria of chemical name, product number, MSDS number, chemical formula and CAS number.

The Elkay Hexacase **compact sample-storage box** is designed for benchtop, refrigerator, freezer or liquid nitrogen storage of cryotubes and microtubes (*Reader Service 125*). This product features a honeycomb design that incorporates the strength and durability of a hexagonal. The Hexacase can accommodate 50 tubes up to 5 cm in height, in individual hexagonal cells with a compact case space of only 0.0035 m³. Constructed of durable autoclavable polypropylene, this case's hinged 'safe-snap' lid closure fully opens for one-handed manipulation. Cases can be stacked vertically and can be fastened together using tongue and groove mating slots located on the sides. The product has reinforced outer walls which increases sample security and base air vents which aid submersion in liquid. There are a variety of bright colours available.

A new 16-page colour brochure highlighting the Masterflex **peristaltic pumps and other new products** is now available from Cole-Palmer (*Reader Service 126*). Pumps with flow rate from as low as 17 ml min⁻¹ up to 30 l min⁻¹ are included. Fluid only comes in contacts the PTFE-tubing —

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

Erratum

The review of the Kristal Clean KC mini-prep kit, *Nature* **376**, 97 (1995), should have stated the manufacturers name as Cambridge Molecular technologies.

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